Environmental Protection Series

Environment Canada Environnement

Report (Canada. Environment Canada)

No: 1 Date: 900700 RM/11

TD 182 R46
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Biological Test Method:

Acute Lethality Test Using *Daphnia* spp.

Report EPS 1/RM/11
July 1990

Canada

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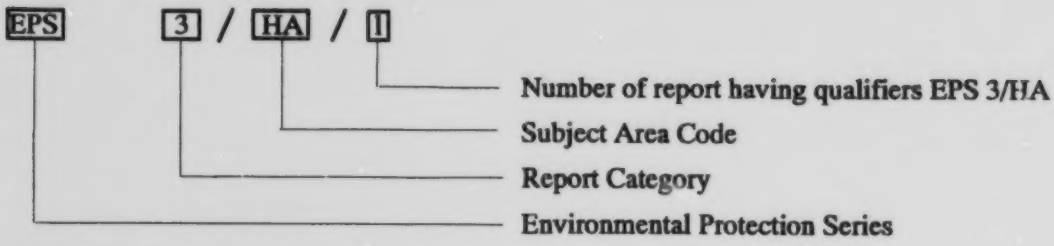

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Biological Test Method:
Acute Lethality Test Using *Daphnia* spp.

Environmental Protection
Conservation and Protection
Environment Canada

Report EPS 1/RM/11
July 1990

91-02027/

Canadian Cataloguing in Publication Data

Main entry under title:

Biological test method : acute lethality test using Daphnia spp.

(Report ; EPS 1/RM/11)
Issued also in French under title: Méthode d'essai biologique, essai de létalité aiguë sur Daphnia spp.
Includes an abstract in French.
Includes bibliographical references.
ISBN 0-662-18076-3 ii.
DSS cat. no. En49-24/1-11E

1. Aquatic biology -- Environmental aspects.
2. Daphnia. 3. Marine pollution.
4. Environmental monitoring -- Canada.
I. Canada. Environmental Protection Directorate.
II. Canada. Environment Canada. III. Series: Report (Canada. Environment Canada) ; EPS 1/RM/11.
IV. Title: Acute lethality test using Daphnia spp.

QH90.57A1 1990 574.92 C90-098684-0

©Minister of Supply and Services Canada 1990
Catalogue No. En 49-24/1-11E
ISBN 0-662-18076-3
BEAUREGARD PRINTERS LIMITED

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Cette publication est aussi disponible en français. Pour l'obtenir s'adresser à :

Publications de la Protection de l'environnement
Conservation et Protection
Environnement Canada
Ottawa (Ontario)
K1A 0H3

Abstract

This document describes methods recommended by Environment Canada for performing acute lethal toxicity tests with daphnids (Daphnia magna *and/or* D. pulex).

General or universal conditions and procedures are outlined for undertaking an acute lethality test using a variety of test materials. Additional conditions and procedures are stipulated which are specific for assessing samples of chemicals, effluents, elutriates, leachates, or receiving waters. Included are instructions on culturing conditions and requirements, sample handling and storage, test facility requirements, procedures for preparing test solutions and test initiation, specified test conditions, appropriate observations and measurements, endpoints, methods of calculation, and the use of reference toxicants.

Résumé

*Le présent document expose les méthodes recommandées par Environnement Canada pour l'exécution d'essais de létalité aiguë sur des daphnies (*Daphnia magna, D. pulex *ou l'une et l'autre espèces).*

Il présente les conditions et méthodes générales ou universelles permettant de réaliser des essais de létalité aiguë sur un large éventail de substances. Il précise d'autres conditions et méthodes propres à l'évaluation d'échantillons de produits chimiques, d'effluents, d'élutriats, de lixiviats ou de milieux récepteurs. Le lecteur y trouvera des instructions sur les conditions et les règles d'élevage des daphnies, la manipulation et le stockage des échantillons, les installations d'essai requises, les méthodes de préparation des solutions d'essai et de mise en route des essais, les conditions prescrites pour les essais, les observations et mesures appropriées, les résultats des essais, les méthodes de calcul et l'utilisation de produits toxiques de référence.

Foreword

*This document is one of a series of **recommended methods** for measuring and assessing the aquatic biological effects of toxic substances. Recommended methods are those which have been evaluated by Conservation and Protection (C&P), and are recommended:*

- *for use in C&P aquatic toxicity laboratories;*
- *for testing which is contracted out by Environment Canada or requested from outside agencies or industry;*
- *in lieu of more specific instructions, such as are contained in regulations; and*
- *as a foundation for the provision of very explicit instructions as may be required in a legal protocol or standard reference method.*

The different types of tests included in this series were selected on the basis of their acceptability for the needs of environmental protection and management programs in Environment Canada. These documents are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on toxic effects of samples of chemicals, effluents, elutriates, leachates, and receiving water.

Mention of trade names in this document does not constitute endorsement by Environment Canada, and other products with similar value are available.

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Glossary

°C . . . . . degree(s) Celsius

$CaCO_3$ . . . . . calcium carbonate

$CaSO_4$ . . . . . calcium sulphate

d . . . . . day

DO . . . . . dissolved oxygen (concentration)

EC_{50} . . . . . Median Effective Concentration

g . . . . . gram

h . . . . . hour

HCl . . . . . hydrochloric acid

H_2O . . . . . water

KCl . . . . . potassium chloride

L . . . . . litre

LC_{50} . . . . . median lethal concentration

LT_{50} . . . . . median lethal time

mg . . . . . milligram

$MgSO_4$ . . . . . magnesium sulphate

min . . . . . minute

mL . . . . . millilitre

mm . . . . . millimetre

N . . . . . Normal

$NaHCO_3$ . . . . . sodium bicarbonate

NaOH . . . . . sodium hydroxide

OD . . . . . outside diameter

PCBs . . . . . polychlorinated biphenyls

SD . . . . . Standard Deviation

SI .....Système international d'unités

TIE .....Toxicity Identification Evaluation

µg .....microgram

YCT .....yeast, Cerophyll™ and trout chow

> .....greater than

< .....less than

≥ .....greater than or equal to

≤ .....less than or equal to

Terminology

Note: All definitions are given in the context of the procedures in this report, and may not be appropriate in another context.

Grammatical Terms

Must is used to express an absolute requirement.

Should is used to state that the specified condition or procedure is recommended and ought to be met if possible.

May is used to mean "is (are) allowed to".

Can is used to mean "is (are) able to".

General Technical Terms

Acclimation means to become physiologically adapted to a particular level of one or more environmental variables such as temperature. The term usually refers to controlled laboratory conditions.

Compliance means in accordance with governmental permitting or regulatory requirements.

Conductivity is a numerical expression of the ability of an aqueous solution to carry an electric current. This depends on the concentrations of ions in solution, their valence and mobility, and on temperature. Conductivity is normally reported in the SI unit of millisiemens/metre, or as micromhos/cm (1 mS/m = 10 μmhos/cm).

Culture is the stock of animals that is raised under controlled conditions to produce test organisms through reproduction.

Daphnid is a freshwater microcrustacean invertebrate, commonly known as a water flea. Species of daphnids include *Daphnia magna* and *D. pulex*.

Dispersant is a chemical substance which reduces the surface tension between water and a hydrophobic liquid (e.g., oil), thereby facilitating the dispersal of the hydrophobic material throughout the water as an emulsion.

Emulsifier is a chemical substance that aids the fine mixing (in the form of small droplets) within water, of an otherwise hydrophobic substance.

Ephippium is an egg case that develops under the postero-dorsal part of the carapace of a female adult daphnid in response to adverse culture conditions. The eggs within are normally fertilized (i.e., sexual reproduction has taken place).

Flocculation is the formation of a light, loose precipitate (i.e., a floc) from a solution.

Hardness is the concentration of cations in water that will react with a sodium soap to precipitate an insoluble residue. In general, hardness is a measure of the concentration of calcium and magnesium ions in water, and is expressed as mg/L calcium carbonate or equivalent.

Lux is a unit of illumination based on units per square metre. One lux = 0.0929 foot-candles and one foot-candle = 10.76 lux.

Monitoring is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality or collection and reporting of information. In the context of this report, it means either the periodic (routine) checking and measurement of certain biological or water-quality variables, or the collection and testing of samples of effluent, elutriate, leachate, or receiving water for toxicity.

Neonate is a newly-born or newly-hatched individual (first-instar daphnid, ≤24-h old).

Percentage (%) is a concentration expressed in parts per hundred parts. One percent represents one unit or part of material (e.g., effluent, elutriate, leachate, or receiving water) diluted with water to a total of 100 parts. The concentrations can be prepared on a volume-to-volume or weight-to-weight basis, or less accurately on a weight-to-volume basis, and are expressed as the percentage of test material in the final solution.

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less than 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

Photoperiod is the duration of illumination and darkness within a 24-h day.

Precipitation means the formation of a solid (i.e., precipitate) from a solution.

Pre-treatment means, in this report, treatment of a sample or dilution thereof, prior to exposure of daphnids.

Salinity is the total amount of solid material, in grams, dissolved in 1 kg of seawater. It is determined after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter or other means (see APHA *et al.*, 1989). It is usually reported in parts per thousand (‰).

Surfactant is a surface-active chemical substance (e.g., detergent) which, when added to a non-aqueous liquid, decreases surface tension and facilitates dispersion of materials in water.

Turbidity is the extent to which the clarity of water has been reduced by the presence of suspended or other matter that causes light to be scattered and absorbed rather than transmitted in straight lines through the sample. It is generally expressed in terms of Nephelometric Turbidity Units.

Terms for Test Materials

Chemical is, in this report, any element, compound, formulation, or mixture of a chemical substance that may enter the aquatic environment through spillage, application, or discharge. Examples of chemicals which are applied to the environment are insecticides, herbicides, fungicides, sea lamprey larvicides, and agents for treating oil spills.

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no test material. The control is used to determine the absence of measurable toxicity due to basic test conditions (e.g., quality of the control/dilution water, health or handling of test organisms).

Control/dilution water is the water used for diluting the test material, or for the control test, or both.

Dechlorinated water is a chlorinated water (usually municipal drinking water) that has been treated to remove chlorine and chlorinated compounds from solution.

Deionized water is water that has been passed through resin columns to remove ions from solution and thereby purify it.

Dilution water is the water used to dilute a test material in order to prepare different concentrations for the various toxicity test treatments.

Distilled water is water that has been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.

Effluent is any liquid waste (e.g., industrial, municipal) discharged to the aquatic environment.

Elutriate is an aqueous solution obtained after adding water to a solid waste (e.g., tailings, drilling mud, dredge spoil), shaking the mixture, then centrifuging or filtering it or decanting the supernatant.

Leachate is water or wastewater that has percolated through a column of soil or solid waste within the environment.

Receiving water is surface water (e.g., in a river) that has received a discharged waste, or else is about to receive such a waste (e.g., it is just upstream from the discharge point). Further description must be provided to indicate which meaning is intended.

Reconstituted water is deionized or glass-distilled water to which reagent-grade chemicals have been added. The resultant synthetic fresh water is free from contaminants and has the desired pH and hardness characteristics.

Reference toxicant is a standard chemical used to measure the sensitivity of the test organisms in order to establish confidence in the toxicity data obtained for a test material. In most instances a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the test material is evaluated, and the precision of results obtained by the laboratory.

Stock solution is a concentrated aqueous solution of the material to be tested. Measured volumes of a stock solution are added to dilution water in order to prepare the required strengths of test solutions.

Upstream water is surface water (e.g., in a stream, river, or lake) that is not influenced by the test material, by virtue of being removed from it in a direction against the current or sufficiently far across the current.

Wastewater is a general term, which includes effluents, leachates, and elutriates.

Toxicity Terms

Acute toxicity is a discernible adverse effect (lethal or sublethal) induced in the test organisms within a short period of exposure to a test material, usually ≤ 48 h for daphnids.

EC_{50} is the median effective concentration (i.e., the concentration estimated to cause a specified non-lethal or lethal effect on 50% of the organisms). The particular effect must be specified as well as the exposure time (e.g., "48-h EC_{50} for immobilization").

Endpoint means the variables (i.e., time, reaction of the organisms, etc.) that indicate the termination of a test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (LC_{50}, EC_{50}, etc.).

Flow-through describes tests in which solutions in test vessels are renewed continuously by the constant inflow of a fresh solution, or by a frequent intermittent inflow.

Immobility is defined as the inability to swim during the 15 seconds which follow gentle agitation of the test solution, even if the daphnids can still move their antennae.

LC_{50} is the median lethal concentration (i.e., the concentration of material in water that is estimated to be lethal to 50% of the test organisms). The LC_{50} and its 95% confidence limits are usually derived by statistical analysis of mortalities in several test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 48-h LC_{50}).

Lethal means causing death by direct action. Death of daphnids is defined here as the cessation of all visible signs of movement or other activity, including second antennae, abdominal legs, and heartbeat as observed through a microscope.

LT_{50} is the time (period of exposure) estimated to cause 50% mortality in a group of organisms held in a particular test solution. The value is best estimated graphically.

Overt means obviously discernible under the test conditions employed.

Static describes toxicity tests in which test solutions are not renewed during the test.

Static replacement describes toxicity tests in which test solutions are renewed (replaced) periodically during the test, usually at

the beginning of each 24-h period of testing. Synonymous terms are "renewal", "batch replacement", and "semi-static".

Sublethal means detrimental to the organism, but below the level which directly causes death within the test period.

Toxicity is the inherent potential or capacity of a material to cause adverse effects on living organisms.

Toxicity Identification Evaluation is a systematic sample pre-treatment (e.g., pH adjustment, filtration, aeration) followed by tests for acute toxicity. This evaluation is used to identify the causative agent(s) which are primarily responsible for acute lethality in a complex mixture.

Toxicity test is a determination of the effect of a material on a group of selected organisms under defined conditions. An aquatic toxicity test usually measures the proportions of organisms affected by their exposure to specific concentrations of chemical, effluent, elutriate, leachate, or receiving water.

Acknowledgements

This document was co-authored by Don McLeay (McLeay Associates Ltd., West Vancouver, B.C.) and John Sprague (J.B. Sprague Associates Ltd., Guelph, Ontario).

Messrs. G. Sergy and R. Scroggins (Environmental Protection, C&P, Environment Canada) acted as Scientific Authorities and provided technical input and guidance throughout the work.

Members of the Inter-Governmental Aquatic Toxicity Group (IGATG) (Appendix A) participated actively in the development and review of this document and are thanked accordingly. Special acknowledgement is made of the technical contributions provided by the IGATG subcommittee members (K. Doe, D. MacGregor, R. Parker, R. Scroggins, G. Sergy, R. Watts, G. Westlake) responsible for initial and final review. The laboratory testing support of Environment Canada (Appendix A) is also acknowledged.

Many useful comments and suggestions were volunteered by the following persons, who reviewed the final draft document: S. Wade (EP, Dartmouth, N.S.); B. Dutka (C&P, Burlington, Ontario); D. Moul (EP, N. Vancouver, B.C.); S. Yee (EP, N. Vancouver, B.C.); J. Vandermeulen (DFO, Dartmouth, N.S.); B. Hobden (DFO, Winnipeg, Man.); J. Servizi (DFO, Cultus Lake, B.C.); G. Joubert (Québec Min. Environnement, Ste-Foy, Québec); D. Poirier (Ont. Min. Environment, Rexdale, Ont.); S. Horvath (B.C. Min. Environment, Vancouver, B.C.); J. Greene (U.S. EPA, Corvallis, Oregon); T. Kovacs (PPRIC, Pointe Claire, Québec); S. Ferguson (PPRIC, Pointe Claire, Québec); S. Gibbons (PPRIC, Pointe Claire, Québec); G. Craig (Beak Consultants Ltd., Brampton, Ont.); R. Evers (B.A.R. Environmental, Guelph, Ont.); P. Chapman (E.V.S. Consultants Ltd., N. Vancouver, B.C.); D. Monteith (B.C. Research Corp., Vancouver, B.C.); and B. Tepper (U.S. Testing Co., Inc., Hoboken, N.J.).

Section 1

Introduction

1.1 Background

No single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection. Delivery of the preventative and remedial measures necessary to manage the environment requires the effective use of a selected battery of well-defined aquatic toxicity tests. Sergy (1987), in consultation with the Inter-Governmental Aquatic Toxicity Group (IGATG; members listed in Appendix A), proposed a set of tests which would be broadly acceptable, and would measure different types of toxic effects in different organisms. The acute lethality test using daphnids was one of several "core" aquatic toxicity tests which was selected to be standardized sufficiently to help meet Environment Canada's testing requirements.

Universal test procedures generically applicable to any acute lethality or mobility inhibition tests with *Daphnia magna* or *D. pulex* performed under controlled laboratory conditions are described in this report. Also presented are specific sets of test conditions and procedures, required or recommended when using the acute lethality test for evaluating different types of materials (namely samples of chemicals, effluents, elutriates, leachates, or receiving waters). Those specific procedures and conditions relevant to the conduct of a test are delineated, and discussed further in explanatory footnotes if necessary. The flowchart in Figure 1 gives a general picture of the universal topics covered in this report, and those specific to testing certain kinds of environmental contaminants. In developing these procedures, an attempt was made to balance scientific, practical, and financial considerations, and to ensure that the results will be accurate and precise enough for the majority of situations in which they will be applied.

The authors assume that the user has a certain degree of familiarity with aquatic toxicity tests. Explicit instructions on every detail that might be required in a specific reference method for regulatory purposes are not provided although this report is intended to serve as a guide to useful methodology for that and other applications.

1.2 Species Description and Historical Use in Tests

Daphnids are freshwater microcrustaceans, commonly referred to as water fleas, belonging to the Order (or Suborder) Cladocera. *D. pulex* attain a maximum length of approximately 3.5 mm, whereas *D. magna* reach 5 to 6 mm. These invertebrate species are a major component of the freshwater zooplankton throughout the world (Hebert, 1978) and may be the dominant herbivore in lakes. *D. magna* is a lake and pond dweller and is restricted to waters in northern and western North America which have hardness values >150 mg/L (Pennak, 1978; Greene *et al.*, 1988). The smaller *D. pulex* resides principally in ponds, quiescent sections of streams and rivers, and to a lesser extent in lakes throughout North America, at locales where water hardness can differ from soft to very hard water (U.S. EPA, 1985a).

The selection of daphnids for routine use in toxicity testing tests by Canadian laboratories is appropriate for a number of reasons:

- in Canada, daphnids are broadly distributed in bodies of fresh water and are present throughout a wide range of habitats;

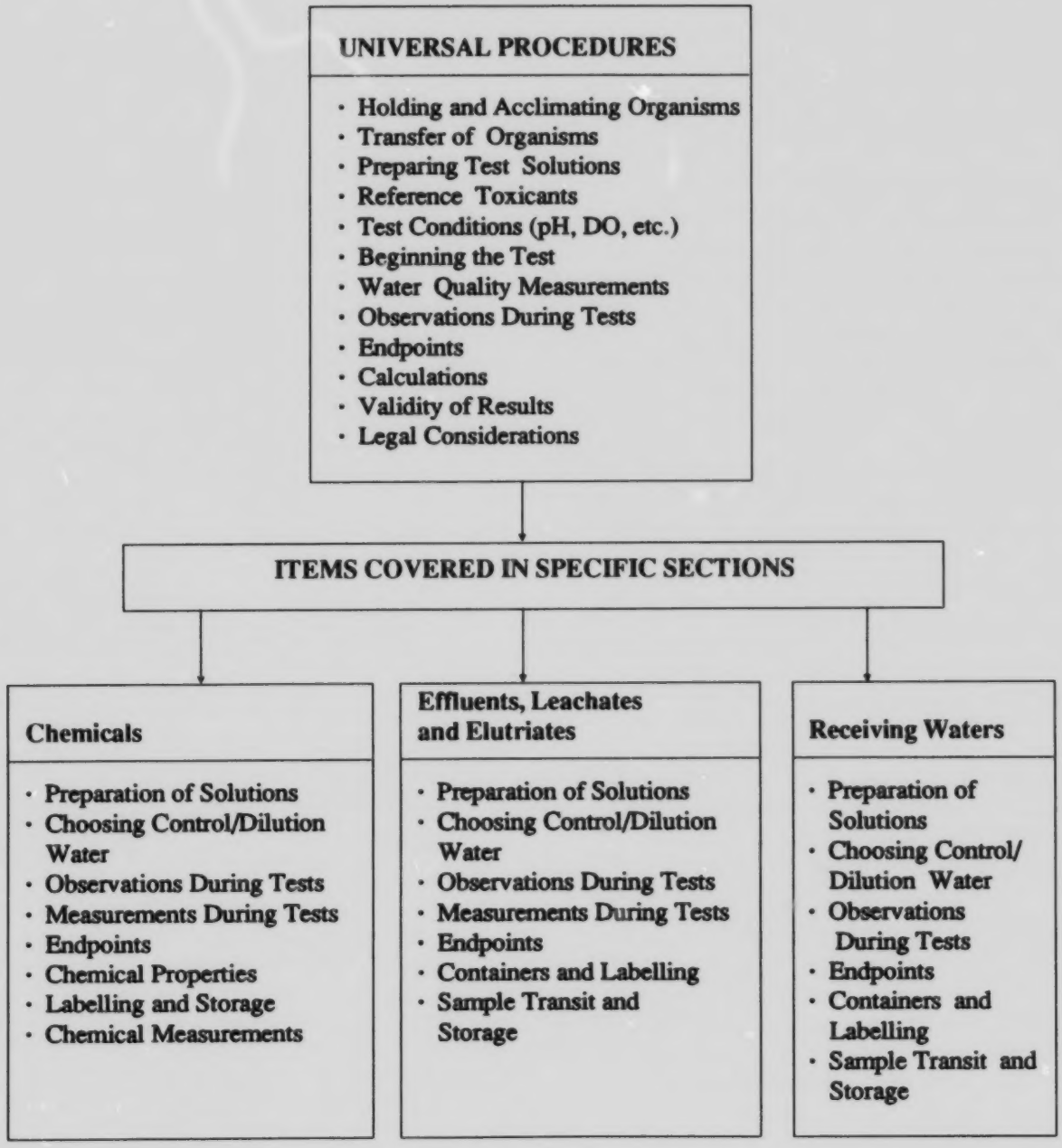

Figure 1 **Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate to Various Types of Materials**

- these organisms are an important link in many aquatic food chains and a significant source of food for juvenile stages of salmonid and other fish species;

- daphnids have a relatively short life cycle and are relatively easy to culture in the laboratory;

- daphnids are sensitive to a broad range of aquatic contaminants, and are widely used as test organisms for evaluating toxicity of chemicals or effluents; and

- the small size of daphnids requires only small volumes of test water, leading to ease of sampling and transporting wastewater samples.

Overseas, daphnids (*D. magna* and/or *D. pulex*) have been used extensively during recent years for evaluating the acute lethality of test materials under defined laboratory conditions (The Netherlands, 1980; OECD, 1981; BHSC, 1982; ISO, 1982; AFNOR, 1983; IGATG, 1986). The United States has also developed standard test procedures with *D. magna* or *D. pulex* for determining the acute toxicity of samples of effluents or chemicals (U.S. EPA, 1982; Plotkin and Ram, 1983; ASTM, 1984; APHA *et al.*, 1989; U.S. EPA, 1985a,b; U.S. EPA, 1987) or for screening of hazardous waste sites for toxicity (Greene *et al.*, 1988). Within Canada, certain provinces have recently drafted or published methodology documents for undertaking acute lethal toxicity tests with effluent samples using daphnids (B.C. MEP, 1988; Poirier *et al.*, 1988; BNQ, 1990). Environment Canada laboratories across Canada (Appendix A) have an established capability for performing acute lethality tests using daphnids as test organisms, although the procedures used at each facility differ somewhat and no "standard" methodology has yet been developed or adopted (Sergy, 1987).

Specific test procedures are sufficiently varied in the existing methodology documents, that comparable results are not assured for different tests with daphnids. Additionally, many procedural considerations are not addressed in those documents (e.g., those related to the modifying influences of test pH or hardness), test endpoints differ, and the application of a defined test has been restricted as to situation, intent, or type of test material. A review of procedural variables and approaches given in existing methodology documents is provided in Appendix B. The purpose of this procedural report is to provide a "standardized" Canadian methodology for undertaking tests of acute lethal toxicity (or acute mobility inhibition) with various test substances, using daphnids as test organisms.

The issues previously discussed have been considered in the development of this methodology report. It has been designed for use with freshwater-acclimated daphnids, test solutions that are essentially fresh water (i.e., salinity ≤10‰) or saline but destined for discharge to fresh water, and fresh water as the dilution and control water. Its application may be varied but includes instances where the impact or potential impact of materials on the freshwater environment is under investigation. Other tests, using other species acclimated to seawater, may be used to assess the impact or potential impact of materials in estuarine or marine environments, or to evaluate test solutions having a salinity >10‰ which are destined for estuarine/marine discharge.

Section 2

Test Organisms

2.1 Species

Either *Daphnia magna* or *Daphnia pulex* (Figure 2) is to be used in a given toxicity test. Choice of species depends on the hardness of the control/dilution water. *D. pulex* may be used at any hardness, and this species is recommended if the hardness of the control/dilution water is less than 80 mg/L. *D. magna* may be used as the test species if the hardness of the control/dilution water is equal to or greater than this value[a].

2.2 Life Stage

Neonate daphnids (≤24 h old) are to be used as test organisms. Neonates are obtained by transferring gravid females from the culture to smaller vessels of culture/control/dilution water, 24 h prior to the start of the test. The addition of food and nutrients (if required) to this water is recommended (see section 2.4.10) in order to increase the percentage of adults which moult and release their broods. It is desirable but not essential to know the age of the females and to use their "middle" broods (i.e., age of females 2 to 4 weeks, see Sections 2.4.3 and 4.2).

2.3 Source

Cultures of *D. magna* or *D. pulex* are available from commercial biological supply houses and from government laboratories. Advice concerning sources of daphnids suitable for conducting aquatic toxicity tests can be obtained by contacting regional Environmental Protection offices (Appendix A).

Very few organisms (20 to 30) are required to start a culture. If taxonomy is in doubt, the daphnid species should be confirmed taxonomically by microscopic examination of the size and number of spines on their postabdominal claws (see Figure 2) (Pennak, 1978; U.S. EPA, 1985a). All organisms used in a test must be from the same culture.

2.4 Culturing

2.4.1 General

Recommended conditions and procedures for culturing daphnids are discussed here and summarized in Table 1. These are intended to allow some degree of inter-laboratory flexibility while standardizing those conditions which, if uncontrolled, might affect the health and performance of the test organism.

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[a] The neonates of *D. magna* are larger and easier to observe in the test solutions. However, this species is found naturally only in hard (>150 mg/L) water (Pennak, 1978) and the use of *D. magna* in soft water solutions may lead to mortality caused by osmotic stress (Greene *et al.*, 1988). Sublethal stress from low hardness might affect resistance to the substance being tested. As *D. pulex* resides naturally in both hard and soft waters, the use of this species for test solutions with hardness values <80 mg/L is recommended here.

Unpublished studies by Environment Canada (EP, Atlantic Region) have demonstrated that at least some laboratory strains of *D. magna* can survive and reproduce normally in water with hardness ≥25 mg/L. However, softer water can cause acute mortalities. A hardness ≥80 mg/L is recommended for culturing and testing *D. magna*.

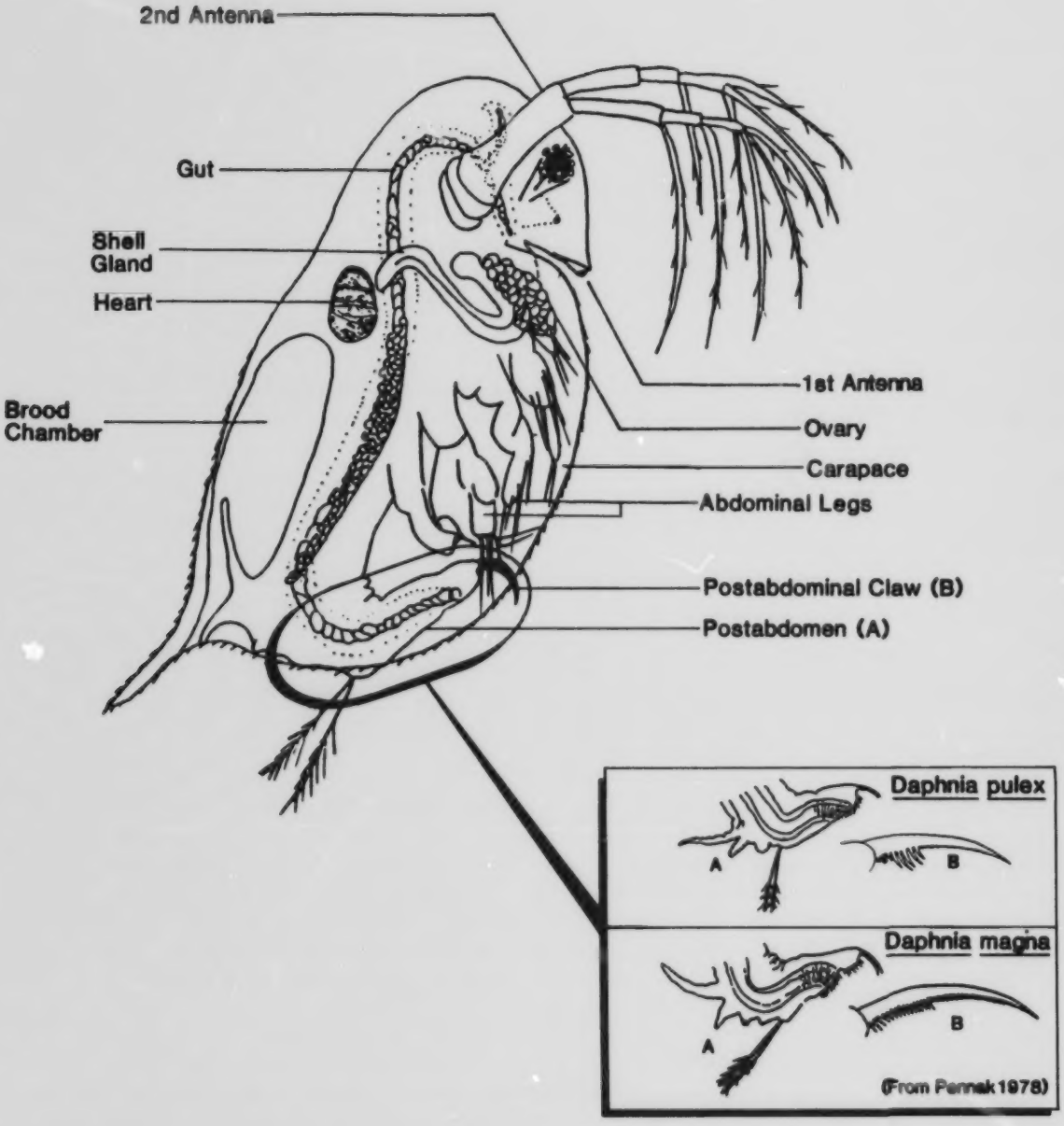

Figure 2 Anatomy of Female *Daphnia* spp.
(from U.S. EPA, 1985a and Poirier *et al.*, 1988)

A number of successful culturing methods are in common use in Canada and elsewhere (U.S. EPA, 1982; ASTM, 1984; U.S. EPA, 1985a; Greene *et al.*, 1988; Poirier *et al.*, 1988; BNQ, 1990) and it is beyond the intent of this document to reiterate the details of this information. Accordingly, persons wishing to initiate daphnid cultures are advised to review the publications cited for specifics regarding proven options for culturing these organisms. The following recommendations and requirements will provide a greater degree of standardization and quality control in yielding daphnids for aquatic toxicity tests.

2.4.2 Facilities

Daphnids are to be reared and maintained in a laboratory facility. The air supply should be free of detectable odours and dust. Ideally, the culturing facility should be isolated from the test facility to prevent or reduce the possibility of culture contamination by volatiles released from test samples and solutions.

Culture vessels and accessories contacting the organisms and culture media must be made of nontoxic materials (e.g., glass, stainless steel, Nalgene™, porcelain, polyethylene). Glass aquaria or wide-mouthed jars ≥3 L are recommended for mass cultures as they permit easy observation of the daphnids.

Materials such as copper, brass, galvanized metal, lead, and natural rubber must not come in contact with culture vessels or media, nor with test samples, test vessels, dilution water, or test solutions. Each culture vessel should be covered to exclude dust and minimize evaporation.

2.4.3 *Management of Cultures*

In the mass cultures of ≥3 L previously mentioned, the water in each culture vessel should be almost completely replaced, at least weekly[b] (U.S. EPA, 1982; Greene *et al.*, 1988). The population of daphnids should be thinned at that time to approximately twenty animals or fewer, per litre. Lower numbers may produce more satisfactory results, and a rearing density of only four adult *D. magna* per litre has been recommended elsewhere, to avoid production of ephippia (Cowgill, 1989). The establishment and maintenance of at least five culture vessels for each test species is desirable[c].

Other approaches have been successful, for example one-litre culture jars have been used, with feeding and renewal of water three times a week. Under such a regime, thinning should be done to the standard value of twenty animals or fewer, per litre.

A recommended alternative is to start new cultures each week, using fresh water and neonates from the previous week's culture. Cultures of three successive ages should be kept on hand (i.e., mothers, daughters, and granddaughters), with replicate containers representing each age. In that way, adults known to be 2 to 5 weeks old would always be available to supply neonates for tests (see Section 4.2).

2.4.4 Lighting

Light intensity should not exceed 800 lux at the water surface, and ideally should be skewed towards the blue end of the spectrum (colour rendering index ≥90) (Buikema, 1973; ASTM, 1984; Poirier *et al.*, 1988). Cool white fluorescent lights are suitable, although other light sources (e.g., full-spectrum

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b If the medium is not replaced at least weekly and if the population density is not reduced to an acceptable level (≤20/L, EP, Atlantic Region), waste products will accumulate which could cause a population crash or the production of males and/or ephippia (Greene *et al.*, 1988). Greene *et al.* (1988) provide details of a siphoning technique to replace 90% of the culture medium.

c Multiple cultures will provide protection against loss of the entire population due to population "crashes" in one or more chambers (U.S. EPA, 1985a).

Table 1 Checklist of Recommended Conditions and Procedures for Culturing Daphnids

Source of daphnids	-	biological supply house or government laboratory; species confirmed by microscopic examination
Water	-	uncontaminated ground, surface, or dechlorinated municipal water, or reconstituted water; water replaced at least weekly and daphnids thinned to 20/L
Temperature	-	within the range 20 ± 2°C, for ≥2 weeks before test
Oxygen/aeration	-	dissolved oxygen 60 to 100% saturation, maintained by aeration (filtered oil-free air) if necessary
pH	-	withing the range 6.0* to 8.5, preferably 6.5 to 8.5
Hardness	-	within 20% of that of control/dilution water, for ≥7 days before test
Water quality	-	temperature, dissolved oxygen, and pH monitored in each culture vessel, preferably daily
Lighting	-	preferably "cool white" fluorescent, ≤800 lux at water surface, 16 ± 1 h light : 8 ± 1 h dark
Feeding	-	algal cultures with or without supplements (e.g., YCT)
Handling	-	minimal, by gentle pouring, siphoning or pipetting
Health criteria	-	brood stock with no ephippia, ≤ 25% mortality during week before test, time to first brood ≤ 12 d, females 2 to 5 weeks old to deliver an average of ≥15 neonates

* Waters with pH >7.4 and hardness ≥80 mg/L should be used for culturing *D. magna*

fluorescent) may be used if the health criteria described in Section 2.4.12 can be met. Photoperiod should normally be a 16 ± 1 h:8 ± 1 h, light:dark cycle[d].

2.4.5 Water

Sources of water for culturing daphnids can be an uncontaminated supply of groundwater, surface water, dechlorinated municipal drinking water, reconstituted water adjusted to the desired hardness (see Sections 2.4.9, 4.1 and 5.3), or "upstream" receiving water taken from a waterbody to be tested. The water supply should consistently and reliably support good survival, health, growth, and reproduction of daphnids. Monitoring and assessment of variables such as hardness, alkalinity, residual chlorine (if municipal water), pH, total organic carbon, conductivity, suspended solids, dissolved oxygen, total dissolved gases, temperature, ammonia nitrogen, nitrite, metals, and total organophosphorus pesticides, should be performed as frequently as necessary to document good water quality.

If municipal drinking water is to be used for culturing daphnids and as control/dilution water, effective dechlorination must rid the water to which daphnids are exposed of any harmful concentration of chlorine. A target value for total residual chlorine in cultures and control/dilution water in test vessels is ≤ 0.002 mg/L (CCREM, 1987). The use of activated carbon (bone charcoal) filters and subsequent ultraviolet radiation (Armstrong and Scott, 1974) is recommended for this purpose[e]. As an alternative, water could be held in reservoirs and aerated strongly for at least 24 h after carbon filtration. In very troublesome cases, algal growth could be encouraged in the reservoirs of aerating water as a means of removing the residual chlorine compounds, with filtration of the water before using it with daphnids.

Culture water must not be supersaturated with gases. In situations where gas supersaturation within the water supply is a valid concern, total gas pressure within water supplies should be frequently checked (Bouck, 1982). Remedial measures (e.g., passing through aeration columns before use or vigorous aeration in an open reservoir) must be taken if dissolved gases exceed 100% saturation. It is not a simple matter to completely remove supersaturation, and frequent checking should be done if the problem is known or suspected to exist.

Water temperature, dissolved oxygen and pH should be monitored for each culture, preferably daily.

2.4.6 Temperature

Water temperature in the cultures should be in the range of 20 ± 2°C. An incubator, water bath, controlled temperature room, or aquarium heater should be used to regulate temperature. If cultures are maintained outside this temperature range, temperature is to be adjusted gradually (≤3°C/day) to a range of 20 ± 2°C and held there for a minimum of two weeks.

2.4.7 Dissolved Oxygen

Water to be used in cultures should be aerated vigorously prior to use to ensure saturation with oxygen and to reduce any supersaturation with gases. Following aeration, the water should be held unaerated for approximately 0.5 h before use in cultures, to allow the escape of air bubbles and assist in the final

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[d] A long (16-h) daily light cycle stimulates asexual reproduction (required for the test), whereas short light periods stimulate sexual reproduction (Buikema *et al.*, 1980).

[e] The addition of thiosulphate or other chemicals to water in order to remove residual chlorine is not recommended. Such chemical(s) could sequester trace metals essential for the health of the organism or, if used as dilution water, could alter sample toxicity.

equilibration of pH, dissolved oxygen, and other gases.

The dissolved oxygen content in cultures should not fall below 60% air saturation. Gentle aeration of each culture using filtered, oil-free compressed air should be provided if necessary to maintain this level of dissolved oxygen (OECD, 1981; U.S. EPA, 1982; Greene *et al.*, 1988; Poirier *et al.*, 1988).

2.4.8 pH

The pH of the water used for culturing daphnids should be within the range of 6.0 to 8.5. Values for pH within the range 6.5 to 8.5 are preferred (Poirier *et al.*, 1988). Water with pH >7.4 (hardness ≥80 mg/L) is normally required for culturing *D. magna*.

2.4.9 Hardness

If natural water (uncontaminated ground, surface, or dechlorinated municipal water) is used for culturing and as control/dilution water, and if *D. magna* is the cultured/test organism, a water hardness within the range 80 to 250 mg/L is recommended.[a] Natural water with a hardness within the range 10 to 250 mg/L is recommended for culturing and testing *D. pulex*. Waters harder than 250 mg/L should not be used except where the effect of water hardness on acute toxicity is under investigation.

Reconstituted water may be used for procedures requiring standardized culture/control/dilution water, or if a suitable supply of uncontaminated natural water is not available. A relatively soft reconstituted water is recommended for each species: total hardness 40 to 48 mg/L for *Daphnia pulex*; and 80 to 100 mg/L for *D. magna* (Sections 4.1, 5.3). These values fit reasonably well with those recommended in other procedural documents (Appendix B), therefore aiding comparisons of toxicity results.

A specific water hardness may be achieved by preparing reconstituted water. Formulae for preparing reconstituted water of a desired hardness (and pH) are given in Table 2 (U.S. EPA, 1985a); other suitable formulae are also available (ISO, 1982). Alternatively, the laboratory supply of uncontaminated ground, surface, or dechlorinated municipal water may be adjusted to the desired hardness by dilution with deionized or distilled water (if too hard) or by the addition of reconstituted hard water or the appropriate ratio and amount of salts (if too soft).

Whether natural or reconstituted water is to be used as the culture/control/dilution water, daphnids must be cultured for a minimum of seven days in water with hardness identical or similar to that which will be used as the control/dilution water in tests.[f] Unless special circumstances or test requirements dictate otherwise, the same water supply should be used for culturing and as the control/dilution water.

2.4.10 Feeding

Feeding is required during culturing of daphnids. The food used should be sufficient and suitable to maintain the test organisms in a nutritional state that will support normal metabolic activity and achieve the health criteria specified in Section 2.4.12. The following suggestions may help to avoid known problems.

A number of proven diets, feeding rations, and schedules for culturing daphnids are described in detail in the literature (U.S. EPA, 1982; ASTM, 1984; U.S. EPA, 1985a; B.C. MEP, 1988; Greene *et al.*, 1988; and

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f Culture water could be reconstituted water of the same source and formulation as that to be used in the test for the control and dilutions, or a natural water with hardness adjusted to within a range ±20% of that of the control/dilution water. Any greater differences in hardness between culture and control/dilution water could result in erroneous test results due to osmotic stress imposed upon the organisms.

Table 2 Preparation of Reconstituted Water of a Desired Hardness (from U.S. EPA, 1985a)

	Reagent Added* (mg/L)				Final Water Quality	
Water Type	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	Hardness**	pH***
very soft	12.0	7.5	7.5	0.5	10 to 13	6.4 to 6.8
soft	48.0	30.0	30.0	2.0	40 to 48	7.2 to 7.6
moderately hard	96.0	60.0	60.0	4.0	80 to 100	7.4 to 7.8
hard	192.0	120.0	120.0	8.0	160 to 180	7.6 to 8.0
very hard	384.0	240.0	240.0	16.0	280 to 320	8.0 to 8.4

* add reagent-grade chemicals to distilled or deionized water

** expressed in mg/L as $CaCO_3$

*** approximate equilibrium pH after aerating for 24 h

Poirier *et al.*, 1988). Success is reported with the feeding and culture methods of Goulden *et al.* (1982). Feeding with laboratory cultures of green algae (*Selenastrum capricornutum* and/or *Chlorella pyrenoidosa*) is common (U.S. EPA, 1982; Poirier *et al.*, 1988) and desirable, and a mixture of two or more species is beneficial, particularly a mixture of green alga(e) and a diatom such as *Nitzschia frustulum* (Cowgill, 1989). *Daphnia* are also reputed to thrive on *Scenedesmus acutus*. Other algae that have been used are *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, and *Ankistrodesmus convolutus* or *A. falcatus*. One source of cultures for these algal species is the University of Toronto Culture Collection.† Some commercial biological supply houses can also supply cultures. Each species of alga must be grown in a suitable culture medium if it in turn is to supply appropriate nutrients to the daphnids.

Alternatively or in conjunction with algal feeding, a prepared feed consisting of yeast and trout chow (U.S. EPA, 1985a; B.C. MEP, 1988; Greene *et al.*, 1988), or yeast, Cerophyll™ and trout chow (YCT) (U.S. EPA, 1989) has been used. A suspension of Tetramin™ tropical fish food has also been used as a food supplement with success.†† However, a completely artificial diet (e.g., based solely on trout chow and/or yeast) is not recommended as it can result in short life of neonates and lower resistance to toxic substances (Cowgill, 1989). Undesirable levels of pesticides or metals and PCBs have been reported in some commercial fish foods, and some investigators therefore prefer using only yeast and/or Cerophyll as supplements for culturing daphnids. General experience of Canadian laboratories suggests that at least one and preferably two or more algal species should be used, perhaps with a supplement of yeast, trout chow and/or Cerophyll.

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†† Personal communication, EP, Atlantic Region.

Formulae for preparing yeast, Cerophyll™ and trout chow (YCT) and algal concentrate (*Selenastrum capricornutum* or a mixture of algal species) as food for daphnid cultures are given in Appendix C (U.S. EPA, 1989). If used, cultures should be fed at the rate of 7 mL YCT and 7 mL algae concentration per litre culture (U.S. EPA, 1989). The algal concentrate and YCT (Appendix C) should be thoroughly mixed by shaking before dispensing. If the YCT is stored frozen, aliquots thawed for use should be stored in a refrigerator (not re-frozen). Unused portions of unfrozen or thawed YCT should be discarded after two weeks. Unused portions of algal concentrate should be stored in the refrigerator and discarded after one month.

Deficiency in vitamin B_{12} or the trace element selenium can result in poor health of daphnids, and these should be routinely added to culture water, at least if reconstituted water is used. Selenium should be added at 2 µg Se/L using sodium selenate (Na_2SeO_4). Insufficient waterborne selenium may cause deterioration of the cuticle of daphnids, shorter life, and failure of progeny to mature and reproduce, according to a citation in Cowgill (1989) of work done by Keating and Dagbuson (1984). Vitamin B_{12} should be added to artificial culture water at 2 µg/L as cyanocobalamin. Stock solutions of vitamin B_{12} are unstable and should not be stored for more than two weeks. Deprivation of this vitamin may cause delayed reproduction, infrequent moulting, and reproductive failure of progeny (Cowgill, 1989; citing work of Keating, 1985).

Final choice of ration and feeding regime is left to the discretion of the individual laboratory based on experience and success in meeting the health criteria specified for cultured organisms (Section 2.4.12).

2.4.11 Handling Organisms

Liquid containing adult daphnids can be poured gently from one container to another without risk of air being trapped under their carapaces or other appreciable damage. Adult daphnids suitable for establishing breeding colonies (i.e., as a source of neonates) may be removed from the cultures by gently pipetting or siphoning them onto an appropriate-sized screen (e.g., 2 x 1 mm mesh). The retained adults are then transferred quickly to another container (see Section 4.2).

Handling and transfer of neonates should be minimal and physical shock to culture vessels must be avoided. Young daphnids are susceptible to air entrapment and should be transferred from one container to another using a glass pipette. A disposable pipette with the delivery end cut off and fire polished to provide an opening of 5 mm is ideal for this purpose (U.S. EPA, 1985a; Greene *et al.*, 1988). The tip of the pipette should be kept under the surface of the water when the daphnids are released (see ASTM, 1984).

2.4.12 Health Criteria

Cultures of daphnids to be used in toxicity tests should meet the following health criteria (Hebert, 1978; Plotkin and Ram, 1983; APHA *et al.*, 1989; B.C. MEP, 1988; Poirier *et al.*, 1988):

- ephippia must not be present in the culture;
- no more than 25% of the brood stock should die within the seven-day period prior to testing, assuming a culture of mixed ages;
- time to first brood should not exceed 12 days; and
- females 2 to 5 weeks old must deliver an average of 15 or more neonates per brood; 20/brood should be easily attainable.

Health of cultures can be monitored closely by frequent checks of times to first brood and average number of neonates per brood (Poirier *et al.*, 1988). To determine these health indices, a few neonates (<24 h old) may be placed in separate glass beakers and maintained as in the main culture. Time to first brood and average number of neonates

per brood are recorded during a 21-day observation period.

A further indication of the health of the culture and its suitability for use in a toxicity test is provided by the test for daphnid sensitivity to a reference toxicant (see Section 4.6).

Section 3

Test System

3.1 Facilities

The test may be performed in a water bath, environmental chamber, or equivalent facility with temperature control (20 ± 2°C). This facility should be well ventilated, and isolated from physical disturbances that may affect the test organisms. As photoperiod control is required (Section 3.2), the facility must be either a separate laboratory or a portion of a laboratory enclosed by curtains (e.g., black plastic sheeting). Dust and fumes should be minimized.

Construction materials and any equipment that may contact the test solutions or control/dilution water into which the organisms will be placed should not contain any substances that can be leached into the solutions or increase sorption of test material[g] (see Section 2.4.2). The laboratory must have the instruments to measure the basic water quality variables (temperature, conductivity, dissolved oxygen, pH) and must be prepared to undertake prompt and accurate analysis of other variables such as hardness and residual chlorine.

3.2 Lighting

Lighting conditions to which test organisms are subjected should be the same as those defined in Section 2.4.4. The photoperiod (normally 16 ± 1 h light:8 ± 1 h dark) is to be timed to coincide with that to which the organisms have been acclimated.

3.3 Test Vessels

Test vessels must be constructed of glass[h] or clear plastic. Borosilicate glass beakers (150- or 250-mL) or glass test tubes (Poirier *et al.*, 1988) may be used. Bags made of inert nontoxic plastic (e.g., Whirl-Pak™) may also be used except for tests with chemicals (see Section 5.2). The vessel chosen should be sufficiently large to ensure that the loading density does not exceed 1 daphnid per 15 mL of test solution.

If volatile materials or wastewaters containing volatiles are to be tested, the test vessels must be closed and there should be little air space above the test solution.

3.4 Control/Dilution Water

The choice of control/dilution water depends on a number of variables including the test material and intent (see Sections 5 to 7), the hardness of the solution(s) to be tested, and the hardness and type of water to which the test organisms have been acclimated (Section 2.4). Accordingly, control/dilution water may be groundwater, unpolluted surface water (river or lake), dechlorinated municipal water from an uncontaminated source, reconstituted water of a desired pH and hardness (see

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g Materials and equipment should be chosen to minimize or eliminate the likelihood of introducing chemical contaminants into the test solutions, causing sublethal toxic effects. Similarly, chosen materials should not cause chemical adsorption or increased volatility, with resultant loss of chemical from solution and error in the test results.

h Glass containers are inert and easily cleaned, and permit the unimpeded observation of test organisms. Adsorption to non-glass containers (e.g., polyethylene, polypropylene, stainless steel) varies markedly among chemicals.

Sections 2.4.9 and 4.1), or a sample of receiving water collected upstream of the influence of the contaminant source, or adjacent to it, but removed from it. If receiving water is to be used, conditions for its collection, transport, and storage should be as described in Section 6.1.

Ideally, the quality of the culture and control/dilution waters should be identical or essentially the same, and the hardness of all test solutions should not differ appreciably from that of the culture water. Notwithstanding, the purpose of the test (e.g., evaluation of receiving waters for the toxic effects of a chemical spill) or problems of practicality, logistics or cost may lead to the selection of a control/dilution water that is less than optimal. The hardness of the control/dilution water must be known before the test is initiated as it may influence the daphnid species to be used for the test (see Sections 2.1 and 2.4.9).

Section 4

Universal Test Procedures

Procedures described in this section apply to all the tests of chemicals and wastewaters described in Sections 5, 6, and 7. All aspects of the test system described in the preceding section must be incorporated into these universal test procedures.

A summary checklist in Table 3 gives recommended universal procedures for acute lethality tests with daphnids, and also procedures for testing specific types of materials.

4.1 Preparing Test Solutions

All test vessels, measurement and stirring devices and daphnid-transfer apparatus must be thoroughly cleaned and rinsed in accordance with good laboratory procedures. Suitable cleaning procedures are given by ASTM (1984).

Reconstituted water with the desired hardness[i] may be prepared for use as the control/dilution water (Sections 2.4.9 and 3.4). Appropriate types and quantities of reagent-grade chemicals to be added to distilled or deionized water in order to provide control/dilution (or culture) water of a specific hardness, alkalinity, and pH are given in Table 2 (ASTM, 1980; U.S. EPA, 1985a). Alternative and successfully-used formulae are given by ISO (1982). The formulae for "soft" and "moderately hard" water are recommended as standard waters for *Daphnia pulex* and *D. magna*, respectively (Section 2.4.9). Reconstituted water should be aerated vigorously in a nontoxic vessel for at least 24 h prior to use (U.S. EPA, 1985a).

Uncontaminated groundwater, natural surface water, or dechlorinated municipal water may also be adjusted to a desired hardness and used as the dilution and control water. In such instances, these waters may be diluted with deionized or distilled water (if too hard) or increased in hardness by the addition of the appropriate ratio and amount of reagent-grade chemicals (Table 2). Hardness of the control/dilution water must be within the range ±20% of that of the water used for culturing the test organisms[i] (Section 2.4.9).

The control/dilution water for daphnid tests is to be adjusted to the test temperature (normally 20 ± 2°C) prior to use. Supersaturation of this water with excess gases must be prevented (see Section 2.4.5).

Before it is used, the control/dilution water should have a dissolved oxygen content 90 to 100% of the air-saturation value. As necessary, the required volume of control/dilution water should be aerated vigorously (oil-free compressed air passed through air stones) immediately prior to use, and its DO content checked to confirm that 90 to 100% saturation has been achieved.

The test concentrations and numbers of test solutions to be prepared will depend on the purpose of the test. Regulatory or monitoring tests of wastewaters or receiving waters may, in some instances, involve the preparation of only one test concentration (e.g., 100% sample) plus a control (see Sections 6 and 7). For tests intended to produce a 48-h LC_{50}, at least five test concentrations plus a control solution (100% dilution water) are to be prepared[i]. An appropriate geometric dilution

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i A preliminary or range-finding test may be conducted prior to the definitive test. It would normally cover a broader concentration range and might be terminated in 24 h or less.

Table 3 Checklist of Recommended Test Conditions and Procedures for Acute Lethality Tests Using *Daphnia* spp.

Universal	
Test type	- static, 48-h duration*
Control/dilution water	- uncontaminated ground, surface, or dechlorinated municipal water; reconstituted water, particularly if a high degree of standardization is desired; "upstream" receiving water to assess toxic impact at a specific location**; dissolved oxygen (DO) content 90 to 100% saturation at time of use
Organisms	- neonates of *Daphnia magna* or *D. pulex*; ≥10 daphnids per concentration; loading density, ≤ one daphnid/15 mL
Temperature	- 20 ± 2°C
Oxygen/aeration	- no aeration during test
pH	- no adjustment if pH of test solution is within the range 6.0 to 8.5***; a second (pH-adjusted) test may be required or appropriate if sample/solution is beyond this range
Lighting	- preferably "cool white" fluorescent, ≤800 lux at surface, normally 16 ± 1 h light : 8 ± 1 h dark
Feeding	- do not feed during test
Observations	- mortalities and atypical behaviour (e.g., immobility, lethargy, circling, floating) observed at beginning and end of test, as a minimum
Measurements	- solution temperature, pH, and DO; at least at beginning and end; conductivity at least at start; hardness to be assessed
Endpoints	- as specified and/or depending on test objectives and material; may be 48-h LC_{50} (requiring 95% confidence limits) or single-concentration test (% mortality at 48 h or earlier; LT_{50})
Reference toxicant	- one or more of zinc sulphate, sodium chloride, or potassium dichromate; static 48-h LC_{50}, determined within fourteen days of test
Test validity	- invalid if control mortality >10% or if >10% of controls show overt stressed behaviour (e.g., immobility)
Chemicals	
Solvents	- to be used only in special circumstances
Concentration	- desirable to measure at beginning and end of exposure, in high, medium, and low strengths and in control(s); if concentrations decline ≥20%, reevaluate by flow-through or static replacement test
Control dilution water	- depends on intent; reconstituted if require high degree of standardization; receiving water if concerned with local toxic impact; otherwise, uncontaminated laboratory water
Effluents and Leachates	
Transport and storage	- transport at ambient temperature (>1°C, <30°C) or at 1 to 8°C if transit time >2 d; sample should not freeze during transit; store in the dark at 1 to 8°C (preferably 4 ± 2°C); the test should begin within three days and must start within five days after sampling
Control/dilution water	- depends on intent; laboratory water, reconstituted water, or "upstream" receiving water for monitoring and compliance
Elutriates	
Transport and storage	- extract within seven days of sample receipt; store in the dark at 1 to 8°C (preferably 4 ± 2°C); test within ten days of sample receipt
Control/dilution water	- as specified and/or depending on intent; reconstituted water if a high degree of standardization is required
Receiving Water	
Transport and storage	- as for effluents and leachates
Control/dilution water	- depends on intent; if studying local impact use "upstream" receiving water as control/dilution water

* Special situations (e.g., unstable chemicals) may require continuous-flow or static replacement tests, or a change in duration.

** If receiving water is used for dilution, an extra control must use the laboratory water in which daphnids were cultured.

*** If pH is outside this range, results may reflect toxicity due to biologically-adverse pH.

series, in which each successive concentration is about 50% of the previous one (e.g., 100, 50, 25, 12.5, 6.3), may be used to assist in precise calculation of the LC_{50} and its 95% confidence limits. Concentrations may be selected from other appropriate dilution series (see Appendix D).

Replicates of each test concentration may be employed if desired. A major advantage of replicates is that two or more estimates of the LC_{50} (or EC_{50}, Section 4.4) may be used in statistical testing of differences from results at other laboratories, other times, other species, etc., assuming that replicates are also available for those other tests. The precision of the estimate of LC_{50} or EC_{50} (not necessarily its accuracy) will also increase as a simple result of using more test organisms. The use of replicate test vessels can be particularly appropriate for evaluation of new chemicals (see Section 5.2).

When receiving water is used as dilution and control water, a second control solution should be prepared using the laboratory water supply to which organisms have been acclimated for two or more weeks. Upstream water cannot be used if it is toxic according to the criteria of the test for which it was intended[j]. In such cases, the laboratory water supply to which daphnids have been acclimated should be used as the control water and for all dilutions. For a given test, the same control/dilution water is to be used for preparing the control and all test concentrations.

Adjustment of sample/solution pH may be necessary (see Section 4.3.2). Solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) at strengths ≤1 *N* should normally be used for all pH adjustments. Some situations (e.g., effluent samples with highly-buffered pH) may require higher strengths of acid or base.

Abernethy and Westlake (1989) provide useful guidelines for adjusting pH. Test solutions or aliquots of samples receiving pH-adjustment[k] should be allowed to equilibrate after each incremental addition of acid or base. An effort should be made to avoid pH overshoots. The amount of time required for equilibration will depend on the buffering capacity of the sample. For effluent samples, a period of 30 to 60 min. is recommended for pH adjustment (Abernethy and Westlake, 1989).

If it is possible that the test sample (e.g., wastewater or receiving water) is very low in hardness (<25 mg/L) and the intended test organism is *D. magna*, sample hardness must be measured before preparing solutions. If the analysis confirms that sample hardness is <25 mg/L, either *D. pulex* should be used for the test[a] or, if *D. magna* are to be used, sample hardness should be adjusted to 25 mg/L. Adjustment should be made by the addition of the appropriate ratio and amount of reagent-grade chemicals suitable for this purpose (see Section 2.4.9). Any sample adjusted for hardness must be thoroughly mixed and its hardness confirmed before use.

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j The use of water other than upstream receiving water as dilution water, will not enable the natural substances or other contaminants within the receiving water to show any effect on the toxicity of the test material. For instance, natural chelating agents such as humic or fulvic acids in the receiving water could bind with the test material and reduce its toxic impact. Conversely, the presence of contaminants in upstream water could increase the toxicity determined for the test material if it was diluted with that water.

k Tests with chemicals or samples of effluent, leachate, or elutriate requiring pH adjustment usually require the separate adjustment of each test solution (including the control). Those with sample(s) of receiving water normally adjust an aliquot of the undiluted sample, prior to preparing the test concentrations.

With the exception of the previous instance, sample hardness is not to be adjusted.

In instances where the influence of solution hardness on sample toxicity is of concern, water hardness should be measured in at least the control, low, and high test concentrations prior to the start of the test. These initial measurements are made on larger volumes of solutions made up in beakers, after any pH adjustments have been made and just before their use to fill the test vessels.

If (and only if) the measured dissolved oxygen concentration at this time in one or more test solutions is <40% or >100% of air saturation, all test solutions should be pre-aerated (prior to daphnid exposure). To achieve this, bubbles of oil-free compressed air should be dispensed through a clean silica-glass air diffuser* or disposable glass pipette. Bubble size should be in the 1 to 3 mm range. The aeration rate should not exceed 7.5 mL/min·L^{-1}, and duration of pre-aeration should be the lesser of 120 minutes and attaining 40% saturation in the highest test concentration (or 100% saturation, if supersaturation is evident). Any pre-aeration of test solutions should be discontinued following this period and the test initiated, regardless of whether 40 to 100% saturation was achieved in all test solutions. Test solutions with sufficient oxygen (≥40% air saturation) should not be aerated.

4.2 Beginning the Test

Neonates (≤24 h old) of either *D. magna* or *D. pulex* are to be used in the test. To obtain the necessary number of young for a test, remove adult females bearing embryos in their brood pouches from the stock cultures 24 h prior to the start of the test. The females should preferably be 2 to 5 weeks old (Section 2.4.3). Transfer these adults to clean glass beakers (400 mL or 1 L) containing control/dilution water and an inoculum of prepared food resulting in the same concentration of food as that used for culturing (see Section 2.4.10). The control/dilution water must have been previously adjusted to the test temperature (20 ± 2°C) and saturated with dissolved oxygen; water within the transfer beakers should not be aerated. Stocking density in these containers should be approximately 10 adults/L or less (Poirier *et al.*, 1988).

The young that are found in the beakers the following day are used for the toxicity test. Five beakers, each containing 10 adults, normally will provide enough neonate daphnids for one toxicity test (Greene *et al.*, 1988).

In obtaining the neonates it is recommended, although not absolutely required, that the age of adults should be known, approximately, in order to avoid using young or senescent females. Daphnids may produce only two or three young in the first brood, then the number may increase to 20 or more, but decline again with senescence. Some authors recommend avoiding the use of the first brood (Goulden *et al.*, 1982), or the first three broods (Cowgill *et al.*, 1985, Cowgill, 1989). Using females of age 2 to 5 weeks is recommended, in order to obtain their "middle" broods. That requires setting up separate culture vessels periodically with individual neonates of known age, or groups of them, and when they reach the appropriate age of 2 to 5 weeks, using them to produce neonates for tests (Section 2.4.3).

Each test vessel placed within the test facility must be clearly coded or labelled to identify the test substance and concentration, date, and time of starting the test. The vessels should be positioned for easy observation of abnormal daphnid behaviour and mortalities. Preferably, the test solutions should be placed in random order (Sprague, 1973).

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* A suitable diffuser, measuring 3.8 x 1.3 cm and fitting 0.5 cm (OD) plastic disposable airline tubing, is available as catalogue item no. AS-1 from Aqua Research Ltd. (P.O. Box 208, North Hatley, Québec, J0B 2C0, phone no. 819-842-2890).

Temperature, dissolved oxygen, and pH of the test solutions should be checked before starting the test and adjusted if required/permitted. The conductivity of each test solution should also be recorded at this time. Options for adjustment of pH are described in Section 4.3.2. Each test solution should be well mixed with a glass rod, Teflon™ stir bar or other non-reactive device, prior to the introduction of test organisms.

Equal numbers of neonate daphnids are to be introduced into each test solution and the control water, at least ten in each treatment, without exceeding the loading density of ≤1 individual per 15 mL. The order of adding daphnids to test vessels should be randomized beforehand.

Transfer of daphnids to the test vessels should be done as quickly as possible, with minimal addition of culture water and minimal stress to the organisms (see Section 2.4.11). The tip of the pipette should be held under the surface of the test solution while releasing organisms[l]. Neonates should be checked immediately upon transfer. Any which are floating or injured should be replaced. Any organisms dropped or injured during transfer are to be discarded. Transfer pipettes should be thoroughly cleaned between transfers if contact has been made with a test solution, or at least rinsed in control/dilution water.

4.3 Test Conditions

The test is to be a 48-h static* one (i.e., no replacement of solutions during test).

The test should be conducted at 20 ± 2°C.

Density in test solutions should not exceed 1 daphnid/15 mL.

Daphnids are not to be fed during the test.

Test solutions are not to be aerated.

The test is not valid if mortality in the control water exceeds 10% or if >10% of the controls show atypical/stressed behaviour (e.g., immobility). In such a case, the apparent health of the culture should be examined by checking reproductive performance (Section 2.4.12) and a search made for problems of chlorine traces in the water (if dechlorinated municipal water is used), nutrition or other aspects of culturing. The search should be continued until control mortality or atypical behaviour is essentially eliminated.

4.3.1 Dissolved Oxygen

If the dissolved oxygen concentration in any test vessel falls below 40% of the air saturation value, or below approximately 5.5 mg/L, the stress from low oxygen could interact with that from toxic substances and make the latter appear more toxic[m]. In some

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l Holding the tip of the pipette below the surface ensures that the neonates are not trapped by surface tension at the air/water interface, and that no bubbles are trapped under the carapace, which would cause them to float.

Although difficult to accomplish, the transfer of culture water to the test solutions via the pipette must be kept to a negligible amount. One useful technique is to have the daphnids in a small amount of water in the pipette, and allow them to swim out while the tip is held in the test water. If dilution of the test concentration by the culture water is a problem, the test solution should initially lack some dilution water and be somewhat less than its final volume. After adding daphnids, a final addition of dilution water would make up the volume to a mark on the vessel, or by an equivalent means.

* Special situations (e.g., volatile or unstable chemicals in solution) may require the use of flow-through or static replacement tests, or a modified test duration.

m The recommended minimum concentration of 40% saturation is above critical levels known to depress daphnid filtration rates. This level of dissolved oxygen is thought to be adequate for the test daphnids.

cases that could be accepted as part of the action of an effluent or other sample. The result for any test vessel becomes invalid, however, if the dissolved oxygen concentration drops below 40% saturation because of improper test procedures such as inadequate dissolved oxygen in the control/dilution water at the time that test solutions are prepared. If procedures are satisfactory (judged by controls) and it is desired to assess the action of toxic substances without the modifying action of low oxygen, a static replacement or flow-through test should be conducted[n] (see Section 5.4).

4.3.2 pH

Toxicity tests should normally be carried out without adjustment of pH. In instances where the chemical, wastewater, or receiving-water sample causes the pH of any test solution to be outside the range 6.0 to 8.5, and it is desired to assess toxic chemicals rather than the lethal or modifying effects of pH, then the pH of the test solutions or sample should be adjusted before adding daphnids, or a second (pH-adjusted) test should be conducted concurrently[o]. For this (second) test, the initial pH of the sample, or of each test solution[k] may (depending on the test objectives) be neutralized (adjusted to pH 7.0) or adjusted to within ±0.5 pH units of that of the control/dilution water, prior to daphnid exposure. Another acceptable approach for this second test is to adjust each test solution including the control, a). to pH 6.0 to 6.5 if the sample has pH <6.0 or causes such a pH; or b). to pH 8.0 to 8.5 if the sample has/causes pH >8.5. Once the test is initiated, the pH of each test solution is monitored (Section 4.4) but not adjusted.

If the purpose of the toxicity test is to better understand the nature of the toxicants in an effluent, elutriate, leachate, or receiving-water sample, pH adjustment is frequently used as one of a number of treatment techniques (e.g., oxidation, filtration, air stripping, addition of chelating agent) for characterizing sample toxicity. Mount and Anderson-Carnahan (1988) list pH adjustment as one of nine "Toxicity Identification Evaluation" (TRE) techniques which, when performed with an acutely toxic aqueous sample, provide the investigator with a useful method for assessing the physical/chemical nature of the toxicant(s) and their susceptibility to detoxification.

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n Flow-through tests use proportional diluters, metering pumps or other apparatus to provide a continuous or frequent intermittent flow of fresh test solution to each test vessel. The temperature and dissolved oxygen content of each test solution is adjusted to acceptable values just prior to dispensing through the apparatus.

o The main reason for not adjusting sample/solution pH is that pH may have a strong influence on the toxicity of a chemical, or substances in a wastewater. For the (generally) low concentrations of waste found in receiving water after dilution, any change from the natural pH, with concomitant modification of toxicity, should be accepted as part of the pollution "package". That leads to the rationale that the pH of test solutions should not be adjusted.

Some chemicals and wastewaters will, however, cause lethal levels of pH in high concentrations of test solution. That is especially true in monitoring or compliance tests with full strength effluent. It seems unlikely that an investigator would be primarily interested in ascertaining whether extreme pH in full-strength effluent was lethal to daphnids, since such a pH would be unrepresentative of what would prevail after even moderate dilution in receiving water. If pH itself were of primary interest, a toxicity test would not seem necessary, since the lethality of extreme pH is well documented and any danger could be much more economically assessed by a simple chemical measurement. The investigator would usually wish to know if toxic substances were present in a wastewater, and determining that requires that any masking by lethal action of pH be eliminated. That rationale leads to the use of pH-adjusted samples or test solutions, where appropriate. The rationale is exactly parallel to standardizing the temperature and dissolved oxygen in the toxicity tests, even if the wastewater itself were 90°C or had low (e.g., <1 mg/L) dissolved oxygen, either of which would, in itself, be rapidly lethal to organisms.

4.4 Test Observations and Measurements

As a minimum, daphnids in each test vessel should be observed at the beginning and end of the test. Additional observations may be warranted during the initial 24-h period, depending on responses observed and the purpose of testing. For instance, investigators desiring time-related information from the test could add observations at 1, 4 and/or 24 hours. In any case, there should be general observations on behaviour and activity of daphnids immediately after starting the test, as they may be temporarily stunned or immobilized by the toxicant. The duration of 48h allows all daphnids to proceed through one moult, at which time they are most sensitive, but does not prolong the test to the point that starvation becomes a major factor.

Daphnids are more easily observed if each test vessel is temporarily illuminated from the side or from below by placing it on a light box or by other means. A black background is also beneficial, and might be combined with advantageous lighting by having one at the side and the other underneath.

For test solutions that are opaque due to colour or suspended solids, observations will be restricted to those made at the termination of the exposure period (48 h). In such instances, the test solution should be poured into shallow dishes (e.g., Petri™ plates) for observation. If observations are still impaired using this procedure, solutions may be poured gently through a fine-mesh netting (e.g., an 0.1-mm aquarium dip net) and the net contents re-suspended in control/dilution water for observations of numbers of surviving test daphnids and their behaviour. Control solution(s) should receive identical treatment.

At each observation time, numbers of dead daphnids in each test vessel should be recorded, and those daphnids removed. Death is indicated by lack of movement of the body, appendages, and heart as observed through a dissecting microscope or other magnifying device. With some narcotic toxicants, daphnids may be completely immobile and the heart rate may slow to 1 or 2 beats per minute. In such a case, beating of the heart becomes the final criterion of death. If careful observation of the heart cannot be made, a *48-h EC_{50} for immobilization* should be reported rather than an LC_{50}. The procedures for estimating an EC_{50} and its confidence limits are the same as for an LC_{50} (Section 4.5) except for the different criterion for effect on an individual daphnid.

Unusual behaviour should be recorded for each test solution at each observation time. For example, immobilization (i.e., inability to swim during the 15 seconds which follow gentle agitation of the test solution, even if the antennae can still be moved), lethargy, floating on the surface, or abnormal rotating or circling should be recorded for each test solution at each observation period. Comparisons of behaviour should be made with the control solution(s).

Measurements must be made and recorded, at least at the beginning and end of exposure, of the pH and dissolved oxygen content in each test concentration including the control(s). Conductivity of each test solution should be measured at the start of the test as a minimum. Temperature should be measured daily in representative solutions.

In consideration of the small volumes in the test vessels, the measurements at the beginning may be made in "extra" test solution made up for the purpose. If there are two or more vessels at the same concentration, final chemical measurements need be done in only one, or may be done by combining the test fluids. Dissolved oxygen should be measured before mixing.

4.5 Test Endpoints and Calculations

Record the mortalities of daphnids at 48 h in each test solution. In multi-concentration tests (see Sections 4.1, 5, 6, and 7), calculate the 48-h LC_{50} and its 95% confidence limits,

and report the method used in those calculations.

Various computer programs for calculating LC_{50} (or EC_{50}) and confidence limits are available and may be used. Stephan (1977) developed a program for estimating LC_{50}s (also applicable to EC_{50}) which uses probit, moving average, and binomial methods, and adapted it for IBM-compatible personal computers. This BASIC program is recommended, and is available for copying onto a user-supplied floppy disk through courtesy of C.E. Stephan, from Environment Canada (address in Appendix A). An efficient micro-computer program for probit analysis is also available from Hubert (1987) and other satisfactory computer and manual methods (APHA *et al.*, 1989; U.S. EPA, 1985a) may be used. Programs using the Trimmed Spearman-Karber method (Hamilton *et al.*, 1977) are available for personal computers but are not recommended here because divergent results may be obtained by operators who are unfamiliar with the implications of trimming off ends of the dose-response data.

The recommended program of C.E. Stephan provides estimates of LC_{50} (or EC_{50}) and confidence limits by each of its three methods, if there are at least two partial mortalities in the set of data. For smooth or regular data, the three results will likely be similar[p], and values from the probit analysis should be taken as the preferred ones and

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p Figure 3 was based on concentrations of 1.8, 3.2, 5.6, 10, and 18 mg/L, causing mortalities of 0, 2, 4, 9, and 10 daphnids, out of 10 in each concentration. The eye-fitted line estimated the LC_{50} as 5.6 mg/L.

Computer programs gave very similar estimates to the graphic one, for the regular data of Figure 3. The LC_{50}s (and 95% confidence limits) were as follows:

Probit analysis of Hubert (1987):		5.56	(4.28 to 7.21)
Stephan (1977)	probit analysis:	5.58	(4.24 to 7.37)
	moving average:	5.58	(4.24 to 7.33)
	binomial:	6.22	(1.8 to 10)
Spearman-Karber method:	0% trim:	5.64	(4.38 to 7.26)
(Hamilton *et al.*, 1977)	10% trim:	5.73	(4.34 to 7.58)
	20% trim:	5.95	(4.34 to 9.80)

The binomial method did not estimate confidence limits, but selected two concentrations from the test as outer limits of a range, within which the true confidence limits would lie.

In fitting a line such as that in Figure 3, relatively more weight should be mentally assigned to points that are near 50% mortality. If successive concentrations yield a series of 0% mortalities, only one such value should be plotted, the one that is "closest to the middle" of the distribution of data. Similarly, only the first of a series of 100% values would be used. The same principle applies to computer programs; only one successive 0% or 100% should be entered; additional ones may distort the estimate of LC_{50}. Logarithmic-probability paper ("log-probit") shown in Figure 3 may be purchased in or ordered through good technical bookstores.

If it is desired to estimate LT_{50}, a graph such as Figure 3 can be plotted using logarithm of time as the horizontal axis. Individual times to death of daphnids could be used but they are seldom available since tests are not inspected continuously. The cumulative % mortality at successive inspections is quite satisfactory for plotting, and an eye-fitted line leads to estimates of confidence limits following the steps listed in Litchfield (1949).

reported. The binomial estimate may differ somewhat from the others. If the results do not include two partial mortalities, the probit and moving average methods do not function, and the binomial method can be used to provide a best estimate of the LC_{50} (or EC_{50}) with conservative (wide) confidence limits.

A check of any computer-derived LC_{50} (or EC_{50}) should be made by examining a plot on logarithmic-probability scales, of percent dead (or percent immobilized, if death not determined) daphnids at 48h for the various test concentrations[P] (see Figure 3, and APHA *et al.*, 1989). Any major disparity between the estimated LC_{50} (or EC_{50}) derived from this plot and the computer-derived LC_{50} (or EC_{50}) must be resolved.

For single-concentration tests, the endpoints depend on the objective of the test. Appropriate endpoints may include: a) determination of % mortality upon exposure of daphnids to the undiluted sample for 48 h; b) % mortalities at various times for toxicity comparisons; or c) measurement of times to death for individual daphnids in each solution.

If successive observations are made (items b or c), the median time to death (LT_{50}) may be estimated if desired, by plotting in similar fashion to Figure 3 except that the horizontal axis is the logarithm of time instead of concentration. The 95% confidence limits may be estimated and compared by carrying the graphic analysis a stage further (Litchfield, 1949). It should be recognized that neither an LT_{50} nor percentage survival at short exposures is a dependable method of judging ultimate toxicity; therefore, comparisons based on those endpoints give only semi-quantitative guidance.

4.6 Reference Toxicant

The routine use of reference toxicant(s) is required to assess, under standardized test conditions, the relative sensitivity of the culture of daphnids and the precision and reliability of data produced by the laboratory (Environment Canada, 1990). Daphnid sensitivity to the reference toxicant(s) should be evaluated upon preparation of a new batch of daphnids for possible use, and within fourteen days before or after the toxicity test or during it.

Criteria used in recommending appropriate reference toxicants for this test may include:

- chemical readily available in pure form;
- stable (long) shelf life of chemical;
- highly soluble in water;
- stable in aqueous solution;
- minimal hazard posed to user;
- easily analyzed with precision;
- toxic at relatively low concentration;
- good dose-effect curve for *Daphnia*;
- known influence of pH on toxicity to *Daphnia*; and
- known influence of water hardness on toxicity to *Daphnia*.

One or more of the following three chemicals (reagent grade) are recommended for use as reference toxicants for this test: sodium chloride; zinc sulphate; potassium dichromate. Daphnid sensitivity should be examined by performing static tests to determine the 48-h LC_{50}, using one or more of these chemicals and the control/dilution water used routinely by the laboratory*. Test conditions (including diluent-water quality) and procedures for undertaking reference toxicants should be as consistent as possible, and as described in this document.

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* Reconstituted water should be used for a greater degree of standardization, particularly if comparison with the results from other laboratories is desired. Standard waters are recommended in Section 4.1. If enough data were obtained with such waters, national standards could be developed for acceptable limits of tests with reference toxicants.

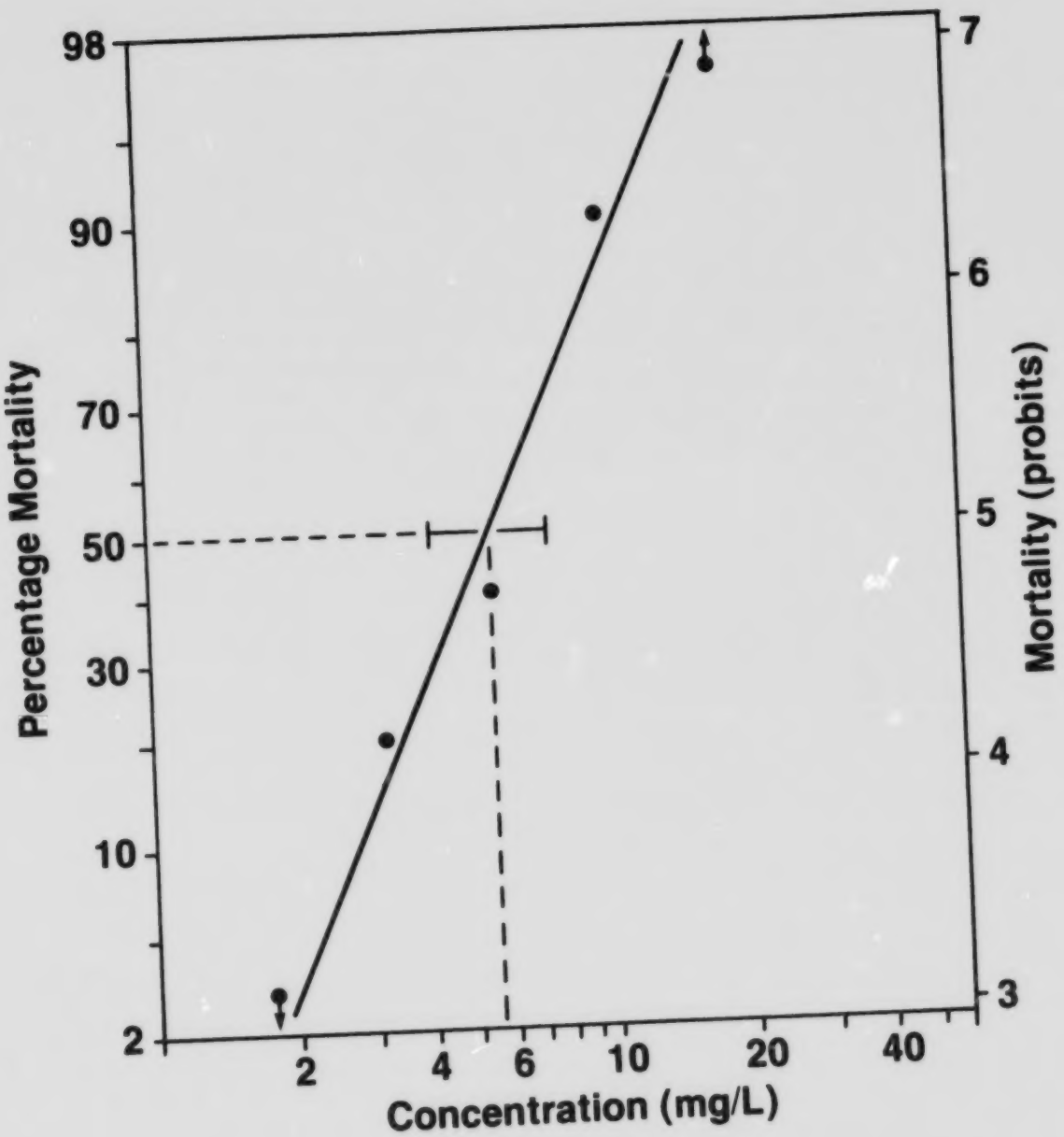

Figure 3 Estimating a Median Lethal Concentration by Plotting Mortalities, or a Median Effective Concentration by Plotting Immobility, Using Logarithmic-probability Paper

In this hypothetical example, ten daphnids were tested at each of five concentrations, and a line was fitted by eye to the results[P]. The concentration expected to be lethal to half the individuals may be read by following across from 50% on the vertical axis (broken line) to the intersection with the fitted line, then down to the horizontal axis, for an estimated LC_{50} or EC_{50} (5.6 mg/L in this instance).

A warning chart (Environment Canada, 1990) should be prepared and updated for each reference toxicant used. Successive LC_{50}s are plotted on this chart and examined to determine whether the results are within ±2 SD for the species, as calculated from the intra-laboratory values obtained in previous tests. The geometric (or arithmetic in instances where data are not normally distributed) mean LC_{50} together with its upper and lower warning limits (±2 SD) are recalculated with each successive LC_{50} until the statistics stabilize (U.S. EPA, 1985a; Environment Canada, 1990). Some guidance on the limits to be expected for potassium dichromate are given by ISO (1982), although unfortunately for 24-h EC_{50}s instead of 48-h values.

If a particular LC_{50} falls outside the warning limits, the sensitivity of the test organisms and the test system are suspect. Inasmuch as this may occur 5% of the time due to chance alone, an outlying LC_{50} does not necessarily mean that the sensitivity of the population of daphnids or the precision of the toxicity data produced by the test laboratory are in question. Rather, it provides a warning that this may be the case. A thorough check of the health of the culture (Section 2.4.12) together with all culturing and test conditions is required at this time. Depending on the findings, it may be necessary to commence the acclimation of a new culture of daphnids and its evaluation (with reference toxicant(s)) before use in toxicity tests.

Concentrations of reference toxicant in all stock solutions should be measured chemically by appropriate methods (e.g., APHA *et al.*, 1989). Upon preparation of the test solutions, aliquots should be taken from at least the control, low, middle, and high concentrations, and analyzed directly or stored for future analysis should the LC_{50} be atypical (outside warning limits). If stored, sample aliquots must be held in the dark at 4 ± 2°C. Zinc solutions should be preserved before storage (APHA *et al.*, 1989). Stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the toxicity test. It is desirable to measure concentrations in the same solutions at the end of the test, after completing biological observations. Calculations of LC_{50} should be based on the average measured concentrations if they are appreciably (i.e., ≥20%) different from nominal ones and if the accuracy of the chemical analyses is reliable.

Concentrations of sodium chloride should be expressed as the weight of the total salt (NaCl) in the water (g/L). That is a slightly different value than the standard measurement of salinity in g/kg or parts per thousand. Zinc sulphate (usually $ZnSO_4 \cdot 7H_2O$, molecular weight 4.3982 times that of zinc) should be used for preparing stock solutions of zinc. Those stock solutions should be acidic (pH 3 to 4), and may be used when prepared or stored in the dark at 4 ± 2°C for several weeks before use. Concentration of zinc should be expressed as mg Zn^{++}/L. Stock solutions of potassium dichromate should be kept in glass-stoppered bottles in the dark, and their concentration should be expressed as mg Cr^{+++}/L. The molecular weight of $K_2Cr_2O_7$ is 2.8290 times that of chromium.

4.7 *Legal Considerations*

Complete and detailed specifications for acute lethality tests undertaken for legal purposes are beyond the scope of this document. It is most important that care be taken to ensure that samples collected and tested with a view to prosecution will be admissible in court. For this purpose, legal samples must be: representative of the substance being sampled; uncontaminated by foreign substances; identifiable as to date, time, and location of origin; clearly documented as to the chain of continuity; and analyzed as soon as possible after collection. Persons responsible for conducting the test and reporting the findings must maintain continuity of evidence for court proceedings (McCaffrey, 1979), and ensure the integrity of the test results.

Section 5

Specific Procedures for Testing Chemicals

This section gives particular instructions for testing chemicals, additional to the procedure in Section 4.

5.1 Properties, Labelling, and Storage of Sample

Information should be obtained on the properties of the chemical to be tested, including water solubility, vapour pressure, chemical stability, dissociation constants, n-octanol:water partition coefficient, and biodegradability. Material data safety sheets should be consulted, if available. If solubility in water is in doubt or problematic, acceptable procedures used previously for preparing aqueous solutions of the chemical should be obtained and reported. Other available information such as structural formula, degree of purity, nature and percentage of significant impurities, and presence and amounts of additives, should be obtained and recorded[q].

Chemical containers must be sealed and coded or labelled (e.g., chemical name, supplier, date received) upon receipt. Storage conditions (e.g., temperature, protection from light) are frequently dictated by the nature of the chemical. Standard operating procedures for chemical handling and storage must be followed.

5.2 Preparing Test Solutions

For testing chemicals, a multiple-concentration test to determine the LC_{50} is usually performed. It may be desirable to have replicates (two or three) of each test concentration, for purposes of evaluating new chemicals. Replicates could be required under regulations for registering a pesticide or similar category of chemical. If a volatile chemical is to be tested, the test vessels should be closed and there should be little air space above the test water.

Test vessels are to be made of glass[h]. Solutions of the chemical to be tested may be prepared either by adding pre-weighed (analytical balance) quantities of chemical to each test vessel as required to give the nominal strengths to be tested[r], or by adding measured volumes of a stock solution. Stock solutions should be prepared by dissolving the test chemical in control/dilution water. For chemicals that do not dissolve readily in water, stock solutions may be prepared using the generator column technique (Billington *et al.*, 1988; Shiu *et al.*, 1988) or, less desirably, by ultrasonic dispersion. The latter technique is not necessarily a complete remedy since the ultrasonics may disperse some of the toxic chemical as an emulsion or fine droplets, and daphnids might take in the droplets selectively, by their filtering activities. Additionally, ultrasonic dispersion can result in variations in the biological availability of the chemical and therefore in its toxicity, due to the production of droplets differing in size and uniformity. Droplets may also migrate towards the surface during the test.

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[q] Knowledge of the properties of the chemical will assist in determining any special precautions and requirements necessary while handling and testing it (e.g., testing in a specially-vented facility, need for solvent). Information regarding chemical solubility and stability in fresh water will also be useful in interpreting test results.

[r] This approach is normally used only for preparing high concentrations or large volumes of test solutions. Otherwise, greater accuracy can be achieved by preparing a stock solution.

Organic solvents, emulsifiers, or dispersants should not be used to increase chemical solubility except in instances where they might be formulated with the test chemical for its normal commercial purposes. If used, an additional control solution should be prepared, with the same concentration of solubilizing agent as that present in the most concentrated solution of the test chemical. Such agents should be used sparingly and should not exceed 0.5 mL/L in any test solution (U.S. EPA, 1985b). If solvents are used, the following are preferred (U.S. EPA, 1985b): dimethyl formamide, triethylene glycol, methanol, acetone, and ethanol.

5.3 *Control/Dilution Water*

For normal intra-laboratory assessment of chemical toxicity, control/dilution water may be reconstituted water or the laboratory supply of uncontaminated ground, surface, or dechlorinated municipal water. In instances where the toxic effect of a chemical on a particular receiving water is to be appraised, sample(s) of the receiving water could be taken from a place that was isolated from influences of the chemical, and used as the control/dilution water[s,t]. Examples of such situations would include appraisals of the toxic effect of chemical spills or intentional applications of chemical (e.g., spraying of a pesticide) on a waterbody. The choice of control/dilution water depends on the intent of the investigator.

If a high degree of standardization is required (for instance, if the toxicity of chemical(s) is to be determined and compared at a number of test facilities), reconstituted water of specified hardness should be used for all dilutions and as the control water[u]. If a single test is to be conducted, a water of low hardness is desirable since it may reveal maximal toxicity of some chemicals. A water hardness of 40 to 48 mg/L is recommended for *Daphnia pulex* and 80 to 100 mg/L for *D. magna* (Section 2.4.9), conforming as well as possible with general use elsewhere (Appendix B). If hardness and other qualities of the dilution water are expected to affect the toxicity of a particular chemical that is to be tested, two or more tests could be run with different reconstituted waters.

5.4 *Test Observations and Measurements*

During solution preparation and at the prescribed observation times during the test, each test solution should be examined for evidence of chemical presence and change (e.g., solution colour and opacity, precipitation or flocculation of chemical). Any observations should be recorded.

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s Contaminants already in the receiving water may add toxicity to that of the chemical or wastewater under investigation. In such instances, uncontaminated control/dilution water (natural or reconstituted) would give a more accurate estimate of the individual toxicity of the spill or spray, but not necessarily of its total impact in the site of interest.

t An alternative (compromise) to using receiving water as dilution and control water is to adjust the pH and hardness of the laboratory water supply (or reconstituted water) to that of the receiving water. Depending on the situation, the adjustment may be to those values measured at a particular time, or to seasonal means.

u Since the hardness, pH and other characteristics of the dilution water can markedly influence the toxicity of the test material, the use of a standard reconstituted water provides results that may be compared in a meaningful way with results from other laboratories.

It is desirable that test solutions be analyzed to determine the concentrations of chemicals to which daphnids are exposed[v]. If chemicals are to be measured, samples should be taken from the high, medium, and low test concentrations and the control solution(s) at the beginning and end of the test, as a minimum. These should be preserved, stored, and analyzed according to the best proven methods for determining the concentration of the particular chemical in aqueous solution.

If chemical measurements indicate that concentrations declined by more than 20% during the test, the acute lethal toxicity of the chemical should be re-evaluated by a test in which solutions are replaced continuously (flow-through test; U.S. EPA, 1982; 1985b) or periodically renewed (static-replacement test; APHA *et al.*, 1989). Toxicity results for any tests in which concentrations are measured should be calculated and expressed in terms of those measured concentrations, unless there is good reason to believe that the chemical measurements are not accurate. In making calculations, each test solution should be characterized by the geometric average measured concentration to which organisms are exposed.

5.5 Test Endpoints and Calculations

The end point for tests performed with chemicals will usually be a 48-h LC_{50}. Accepted procedures for calculating the LC_{50} and its 95% confidence interval are given in Section 4.5.

If a solvent control is used, the test is rendered invalid if mortality in this control (or in the untreated control water) exceeds 10%. The test is also invalid if >10% of the daphnids in either control exhibit atypical/ stressed behaviour (e.g., immobility).

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v Such analyses need not be undertaken in all instances, due to cost, analytical limitations, or previous technical data indicating chemical stability in solution under conditions similar to those in the test.

Chemical analyses are particularly advisable if (U.S. EPA, 1985b): the test solutions are aerated; the test material is volatile, insoluble, or precipitates out of solution; the test chemical is known to sorb to the material(s) from which the test vessels are constructed; or a flow-through system is used. Some situations (e.g., testing of pesticides for purposes of registration) may require the measurement of chemical concentrations in test solutions.

Section 6

Specific Procedures for Testing Effluent, Elutriate, and Leachate Samples

This section gives particular instructions for testing samples of effluents, elutriates, and leachates, in addition to the procedure in Section 4.

6.1 *Sample Labelling, Transport, and Storage*

Containers for transportation and storage of samples of effluents, elutriates, and leachates must be made of nontoxic material (e.g., glass, polyethylene, or polypropylene). The containers must either be new or thoroughly cleaned, and rinsed with uncontaminated water. They should also be rinsed with the sample to be collected. Containers should be filled to minimize any remaining air space.

Upon collection, each sample container must be filled, sealed, and labelled or coded. Labelling should include at least sample type, source, date and time of collection, and name of sampler(s). Unlabelled or uncoded containers arriving at the laboratory should not be tested. Nor should samples arriving in partially- filled containers be routinely tested, since volatile toxicants escape into the air space. However, if it is known that volatility is not a factor, such samples might be tested at the discretion of the investigator.

Testing of effluent and leachate samples should commence as soon as possible after collection. The test should begin within three days and must commence no later than five days after termination of sampling. Samples collected for extraction and subsequent testing of the elutriate should be tested within ten days of receipt. Elutriates should be tested within three days of sample preparation or as specified.

It is desirable to refrigerate samples of effluent and leachate upon collection and during their transport. In situations where this is impractical (e.g., shipment of large volumes of sample), effluent and leachate samples may be held at ambient temperature during transport. However, when ambient temperatures are extreme (i.e., >30°C or <1°C) or when transit times greater than two days are anticipated, the temperature of the samples should be controlled (1 to 8°C) in transit.

Samples should not freeze during transport. Upon arrival at the laboratory, effluent, and leachate samples may be adjusted immediately or overnight to 20 ± 2°C, and testing commenced. If more prolonged sample storage is needed, sample containers should be stored in darkness at 1 to 8°C and preferably at 4 ± 2°C.

Unless otherwise specified, temperature conditions during transportation and storage of elutriates, as well as samples intended for aqueous extraction and subsequent testing of the elutriate, should be as indicated previously.

6.2 *Preparing Test Solutions*

Samples in the collection containers must be agitated thoroughly just prior to pouring to ensure the re-suspension of settleable solids. Sub-samples (i.e., a sample divided between two or more containers) must be mixed together to ensure their homogeneity. If further sample storage is required, the composited sample (or a portion thereof) should be returned to the sub-sample containers and stored (Section 6.1) until used.

6.3 *Control/Dilution Water*

Tests conducted with samples of effluent or leachate for monitoring and regulatory compliance purposes should use either the natural or reconstituted water used for culturing the daphnids, or a sample of the receiving water[s,t] as the control/dilution water. Since results could be quite different for the three sources of water, the objectives of the test must be decided before a choice is made. Shipping difficulties and costs should also be considered, since the use of receiving water for dilutions and as control water greatly increases the volume of liquid to be shipped.

The use of receiving water as the control/dilution water may be desirable in certain instances where site-specific information is required regarding the potential toxic impact of an effluent, leachate, or elutriate on a particular receiving water[s,t]. Conditions for the collection, transport, and storage of such receiving-water samples should be as described in Section 6.1.

If a sample of upstream receiving water is to be used as dilution and control water, a separate control solution should be prepared using the laboratory water supply in which daphnids have been cultured during the preceding two or more weeks. Daphnid survival and behaviour (Section 4.4) in the laboratory control water should be compared to that shown in the sample of receiving water[w].

Tests requiring a high degree of standardization may be undertaken using reconstituted water of a specified hardness (Table 2) as the dilution and control water[u]. Such use could be appropriate in a program to assess the toxicity of a given sample or series of samples, by comparing estimates derived at a number of laboratories or at a single laboratory which had variable water quality. The intent of using reconstituted water would be minimization of modifying influences of differing chemical conditions in dilution water.

6.4 *Test Observations and Measurements*

Colour, turbidity, odour, and homogeneity (i.e., presence of floatable material or settleable solids) of the effluent, leachate, or elutriate sample should be observed at the time of preparing test solutions. Precipitation, flocculation, colour change, release of volatiles or other reactions upon dilution with water should be recorded. Changes in appearance of test solutions during the test (e.g., foaming, settling, flocculation, increase or decrease in turbidity, colour change) should also be noted.

For effluent samples with appreciable solids content, it is desirable to measure total suspended and settleable solids (APHA *et al.*, 1989) upon receipt, as part of the overall description of the effluent, and as items that may influence the results of the toxicity test.

6.5 *Test Endpoints and Calculations*

Tests for monitoring and compliance with regulatory requirements should normally include, as a minimum, one or more undiluted portions of the sample and one or more control solutions. Depending on specified regulatory requirements, tests for compliance may be single-concentration tests (100% wastewater unless otherwise specified) or tests to measure the 48-h LC_{50} (see Section 4.5).

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[w] A comparison of daphnid behaviour (e.g., mobility, circling, floating) and survival in this control water versus the receiving-water sample collected upstream will distinguish any overt toxic responses that may be attributable to contaminants within the upstream water.

Tests undertaken for monitoring effluent, leachate, or elutriate toxicity may also be single-concentration tests to measure percent mortality at 48 h, tests to estimate the LT_{50} at full strength and/or dilutions of the sample, or tests to estimate the 48-h LC_{50}. The end point will depend on a number of considerations including: the objectives of the monitoring program, compliance requirements, test costs, and past history of daphnid survival in the undiluted wastewater.

Toxicity tests conducted for other purposes (e.g., determination of in-plant sources of toxicity, treatment effectiveness, effects of process changes on toxicity) may, depending on the study objectives, be single-concentration tests (100% or an appropriate dilution, plus a control), or multiple-concentration tests. Single-concentration tests are often cost-effective for determining the presence or absence of acute lethal toxicity, or for screening a large number of samples for relative toxicity. Endpoints for these tests would depend on the objectives of the undertaking, but could include arbitrary "pass" or "fail" ratings, percent mortality of daphnids at 48 h or an earlier time such as 24 h, or times to death for individual daphnids in each solution. Items in Section 4.5 are relevant here.

Section 7

Specific Procedures for Testing Receiving-water Samples

Instructions for testing samples of receiving waters, additional to those provided in Section 4, are given here.

7.1 Sample Labelling, Transport, and Storage

Procedures for the labelling, transportation, and storage of samples of receiving waters should be as described in Section 6.1. Testing of samples should commence as soon as possible after collection. The test should begin within three days and must commence no later than five days after termination of sampling.

7.2 Preparing Test Solutions

Samples in the collection containers should be agitated before pouring to ensure their homogeneity. Compositing of sub-samples should be as described in Section 6.2.

7.3 Control/Dilution Water

For receiving-water samples collected in the vicinity of a wastewater discharge, chemical spill, or other point-source of possible contamination, "upstream" water may be sampled concurrently and used as control water and diluent for the downstream samples[s,t]. This control/dilution water should be collected as close as possible to the contaminant source(s) of concern, but upstream of the zone of influence or outside it.

If "upstream" water is used as control/dilution water, a separate control solution should be prepared using the source of water in which the test organisms were cultured for two or more weeks. The acute survival and behaviour (Section 4.4) of test daphnids in this laboratory control water should be compared to that for daphnids held under identical conditions in the upstream control water[w]. If mortalities or signs of distress are evident for test organisms held in the upstream control water and if dilutions of downstream water are being prepared for testing (toxicity anticipated), a separate set of dilutions should be prepared using the laboratory water supply in which daphnids have been reared. Investigators anticipating this eventuality should collect sufficient volumes of receiving water to permit these additional dilutions to be prepared.

Logistic constraints, expected toxic effects or other site-specific practicalities may prevent or rule against the use of upstream water as the control/dilution water. In such cases, the laboratory water supply used for culturing the daphnids should be used as control water and for all dilutions. It could be adjusted to partially simulate upstream water[t].

7.4 Test Observations and Measurements

Observations and measurements of test samples and solutions for colour, turbidity, foaming, precipitation, etc. should be made as described in Section 6.4, both during preparation of test solutions and subsequently during the tests. These are in addition to the primary observations on the organisms, described in Section 4.4.

7.5 *Test Endpoints and Calculations*

Endpoints for tests with samples of receiving water should be consistent with the options and approaches identified in Sections 4.5 and 6.5.

Tests for monitoring and compliance purposes should normally include, as a minimum, one or more undiluted portions of

the sample and one or more control solutions. Endpoints for tests with receiving-water samples may be restricted to a determination of the percentage mortality of daphnids at 48 h in the undiluted sample, together with time-to-death data where applicable.

In instances where toxicity of receiving-water samples is likely and information is desired concerning the degree of dilution necessary to permit the short-term survival of daphnids, a test to determine the 48-h LC_{50} should be conducted. One or more undiluted (100% sample) concentrations and at least four dilutions should be included in this test, together with one or more control solutions. Assuming that data permit, the LC_{50} and its 95% confidence limits should be computed.

Section 8

Reporting Requirements

The test report should describe the materials and methods used, as well as the test results. The reader should be able to establish from the report whether the conditions and procedures rendered the results acceptable for the use intended.

Procedures and conditions that are common to a series of ongoing tests (e.g., routine toxicity tests for monitoring or compliance purposes) and consistent with specifications in this document may be referred to by citation or by attaching a general report which outlines standard laboratory practice. Where choices exist, the approach selected should be specified. The general report should convey the procedural information included in Sections 8.2 to 8.6. An individual test report giving the findings should contain the information indicated in Sections 8.1 and 8.7. Specific monitoring programs may require selected items (e.g., procedures and results for tests requiring pH and/or hardness adjustments) in the test report. Other details pertinent to the conduct and findings of the test, which are not conveyed by the reports, should be kept on file by the test laboratory, so that the appropriate information can be provided if an audit of the test is required.

8.1 Test Material

- sample type, source, and description (chemical, effluent, elutriate, leachate, or receiving water; sampling location and method; specifics regarding nature, appearance and properties, volume and/or weight);
- information on labelling or coding of the test material;
- details on manner of sample collection, transport, and storage (e.g., batch, grab or composite sample, description of container, temperature of sample upon receipt and during storage);
- identification of person(s) collecting and/or providing the sample; and
- dates and times for sample collection, receipt at test facility, and start of definitive test.

8.2 Test Organisms

- species and source;
- description of culturing conditions and procedures (facilities, lighting, water source and quality, water pre-treatment, aeration conditions and apparatus, breeding method including frequency of water exchange and procedure for replacement, methods of handling organisms, temperature range during culturing, age of culture, food type, ration and frequency of feeding);
- estimated percent mortality in culture during seven days preceding test;
- records of time to first brood and average number of neonates per brood;
- procedures and methods used to obtain neonate test organisms from breeding adults; and
- age of test organisms at beginning of test.

8.3 Test Facilities and Apparatus

- name and address of test laboratory;

- name of person(s) performing the test;
- description of systems for regulating light and temperature within test facility; and
- description of test vessels (size, shape, and material).

8.4 Control/Dilution Water

- type(s) and source(s) of water used as control and dilution water;
- type and quantity of any chemical(s) added to control or dilution water;
- sampling and storage details if the dilution water was "upstream" receiving water;
- water pre-treatment (adjustment of temperature, hardness, pH, de-gassing, aeration rate, and duration, etc.); and
- measured water-quality variables before and/or at time of commencement of toxicity test.

8.5 Test Method

- mention of method used if standard (e.g., this document);
- design and description if specialized procedure (e.g., periodic or continuous replacement of solutions) or modification of standard method;
- method of preparing stock and test solutions of chemicals;
- any chemical analyses of test solutions and reference to analytical method(s) used;
- use of preliminary or range-finding test; and
- frequency and type of observations made during test.

8.6 Test Conditions

- number, concentration, volume, and depth of test solutions including controls;
- number of organisms per test solution and per 10-mL volume;
- photoperiod, light source, and intensity at surface of test solutions;
- statement concerning aeration (rate, duration, manner of application) of test solutions prior to daphnid exposure;
- description of any test solutions adjusted for pH or hardness, including procedure and timing;
- conditions and procedures for measuring the 48-h LC_{50} of the reference toxicant;
- any chemical measurements on test solutions (e.g., hardness, concentration of chemical suspended solids); and
- temperature, pH, dissolved oxygen (mg/L and % saturation) and conductivity as monitored in each test solution.

8.7 Test Results

- appearance of test solutions and changes noted during test;
- daphnid behaviour; number and percentage showing mortality or immobility in each test solution including control at each observation time; number and percentage of controls showing atypical/stressed behaviour;
- results for range-finding test (if conducted);
- any 48-h LC_{50} or LT_{50} determined (with associated 95% confidence limits), and reference to the statistical method used for their calculation; and
- the 48-h LC_{50} and 95% confidence limits for the reference toxicant(s), determined within 14 days of the test using the same culture of daphnids as in the test, together with the mean value ± 2 SD for the same reference toxicant, as derived previously at the test facility.

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Appendix A

List of Members of the Inter-Governmental Aquatic Toxicity Group and Conservation & Protection Regional and Headquarters' Office Addresses

Members of the Inter-Governmental Aquatic Toxicity Group*

Federal**

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V7M 3H7

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* A BASIC computer program for calculating LC_{50}s is available for copying onto a formatted 13-cm IBM-compatible floppy disk supplied by the user, by contacting the Aquatic Toxicity Laboratory at this address.

Appendix B

Review of Procedural Variations for Undertaking Acute Lethality Tests Using *Daphnia* spp. (as specified in Canadian, U.S., and International methodology documents)*

1. Type of Test Material

Document	Test Material
APHA *et al.* 1985	effluent
ISO 1982	effluent or other soluble substance
ASTM 1984	effluent
OECD 1981	chemical
U.S. EPA 1982	chemical
Plotkin/Ram 1983	chemical or effluent
The Netherlands 1980	chemical or effluent
BHSC 1982	chemical
U.S. EPA 1985b	chemical
Greene *et al.* 1988	elutriate or receiving water
B.C. MEP 1988	effluent
Poirier *et al.* 1988	effluent

2. Organism, Test Type, and Duration

Document	Test Organism	Life Stage (h)	Test Type	Duration (h)
APHA *et al.* 1985	*D. magna*	neonate(<24)	St[a]	120
ISO 1982	*D. magna*	neonate(6 to 24)	St	24
ASTM 1984	*D. magna/pulex*	neonate(<24)	St	48
OECD 1981	*Daphnia sp.*	neonate(<24)	St	24
U.S. EPA 1982	*D. magna/pulex*	neonate(≤24)	St,Ft[b]	48
Plotkin/Ram 1983	*D. magna/pulex*	neonate(<24)	St	48
The Netherlands 1980	*D. magna*	neonate(<24	St	48
BHSC 1982	*D. magna/pulex*	neonate(6 to 24)	St	48
U.S. EPA 1985b	*D. magna/pulex*	neonate(<24)	St, Ft	48
Greene *et al.* 1988	*D. magna/pulex*	neonate(2 to 24)	St	48
B.C. MEP 1988	*D. magna/pulex*	neonate(<48)	St	48
Poirier *et al.* 1988	*D. magna*	neonate(≤24)	St	48

[a] St = Static test

[b] Ft = Flow-through (continuous-flow) test

* Based on documents available to the authors in August 1988.

3. Culture and Acclimation Conditions

Document	Water Source				Temperature (°C)	Hardness	Aeration
APHA *et al.* 1985	Rc[a]	NW[b]		RW[c]	20	NI[e]	none
ISO 1982		NW	DW[d]		20 ± 2	NI	none
ASTM 1984				RW	20 ± 4	NI	if needed, DO ≥40%
OECD 1981	Rc	NW			19 ± 1	NI	culture aerated
U.S. EPA 1982	Rc	NW	DW	RW	20 ± 1	NI	if needed, DO ≥60%
Plotkin/Ram 1983	Rc	NW			21 ± 2	NI	gentle aeration
The Netherlands 1980	Rc				19 ± 1	NI	culture aerated
BHSC 1982	Rc	NW			NI	NI	NI
U.S. EPA 1985b	NI				NI	NI	NI
Greene *et al.* 1988	Rc				22 ± 4	*D. magna* 80 to 100 *D. pulex* 40 to 48	gentle if needed for DO≥ 6 mg/L
B.C. MEP 1988	Rc	NW			NI	*D. magna* 110 *D. pulex* 50	NI
Poirier *et al.* 1988	DW or other				20 ± 1	120 to 250	gentle aeration

a Rc = Reconstituted water
b NW = Natural surface or groundwater, uncontaminated source
c RW = Receiving water
d DW = Dechlorinated municipal water
e NI = Not indicated

4. Lighting Conditions During Culturing

Document	Photoperiod (L:D)	Intensity (lux)	Type	Dawn/Dusk
APHA *et al.* 1985	NI	NI	NI	NI
ISO 1982	NI	NI	NI	NI
ASTM 1984	16h:8h	≤800	wide-spect. Fl.	NI
OECD 1981	8h:16h	NI	NI	NI
U.S. EPA 1982	16h:8h	NI	NI	15 to 30 min.
Plotkin/Ram 1983	12h:12h	ambient lab.	NI	NI
The Netherlands 1980	8h:16h	NI	NI	NI
BHSC 1982	NI	NI	NI	NI
U.S. EPA 1985b	NI	NI	NI	NI
Greene *et al.* 1988	16h:8h	540 to 1080	NI	NI
B.C. MEP 1988	14h:10h or natural	NI	NI	NI
Poirier *et al.* 1988	16h:8h	≤800	cool-white Fl.	NI

5. Feeding Conditions During Culture and Testing

Document	Feeding of Culture	Feeding During Test
APHA *et al.* 1985	manure + yeast, alga or grass	no
ISO 1982	meat extract + glucose or alga	NI
ASTM 1984	natural or artificial foods	no
OECD 1981	alga (*Chlorella* sp.)	no
U.S. EPA 1982	alga or other foods	no
Plotkin/Ram 1983	trout chow + yeast	yes (added to sol'n.)
The Netherlands 1980	alga (*Chlorella* sp.)	no
BHSC 1982	NI	no
U.S. EPA 1985b	NI	NI
Greene *et al.* 1988	trout chow, alfalfa, yeast + alga	no
B.C. MEP 1988	trout chow, yeast + alga	no
Poirier *et al.* 1988	mixed algal culture	no

6. Test Conditions

Document	Container	Test volume (mL)	No. of *Daphnia* /vessel	mL of solution /Daphnia	No. of repli-cates
APHA *et al.* 1985	125-mL beaker[a]	100	10	10	3
ISO 1982	beaker/t.t.[b]	NI	≤20	≥2	NI
ASTM 1984	250-mL beaker	200	5	40	4
OECD 1981	NI	NI	5	2	4
U.S. EPA 1982	250-mL beaker	200	10	≥25	≥2
Plotkin/Ram 1983	1-L beaker	200 to 400	10	20 to 40	2
The Netherlands 1980	0.25, 1-L beaker	250 to 1000	25	10 to 40	2
BHSC 1982	NI	NI	5	2	4
U.S. EPA 1985b	250-mL beaker	200	10	20	2
Greene *et al.* 1988	100-mL beaker	50	10	5	3
B.C. MEP 1988	disp. Whirlpacks™	NI	10	10	2
Poirier *et al.* 1988	25 x 100 mm t.t.	50	3	15	1

a beaker = glass beaker
b t.t. = glass test tube

7. Characteristics of Control/Dilution Water

Document	Water Type				Hardness (mg/L)	pH	Minimum DO
APHA *et al.* 1985	Rc[a]	NW[b]		RW[d]	NI	NI	NI
ISO 1982	Rc				250 ± 25	7.8 ± 0.2	>80% sat'n.
ASTM 1984	Rc	NW		RW	NI	NI	NI
OECD 1981	Rc	NW			NI	NI	>80% sat'n.
U.S. EPA 1982	Rc	NW	DW[c]	RW	NI	NI	≥90% sat'n.
Plotkin/Ram 1983	Rc	NW			170 ± 10	NI	NI (aerated)
The Netherlands 1980	Rc				NI	NI	100% sat'n.
BHSC 1982	Rc	NW			NI	NI	NI
U.S. EPA 1985b	Rc	NW			40-48	NI	NI
Greene *et al.* 1988	Rc				*D. magna* 80 to 100 *D. pulex* 40 to 48	NI	≥60% sat'n.
B.C. MEP 1988	Rc	NW			D. *magna* 110 *D. pulex* 50	NI	≥90% sat'n.
Poirier *et al.* 1988	DW or other				120 to 250	6.5 to 8.5	NI

[a] Rc = Reconstituted water
[b] NW = Natural surface or groundwater, uncontaminated source
[c] DW = Dechlorinated municipal water
[d] RW = Receiving water

8. Lighting Conditions During Test

Document	Photoperiod (L:D)	Intensity (lux)	Type	Dawn/Dusk (min.)
APHA *et al.* 1985	NI	NI	NI	NI
ISO 1982	24-h darkness	-	-	-
ASTM 1984	16h:8h	≤800	wide-spec.Fl.	NI
OECD 1981	24-h darkness	-	-	-
U.S. EPA 1982	16h:8h	NI	NI	15 to 30
Plotkin/Ram 1983	12h:12h	540 to 1080	NI	NI
The Netherlands 1980	10h:14h	NI	NI	NI
BHSC 1982	optional	NI	NI	NI
U.S. EPA 1985b	16h:8h	NI	NI	15 to 30
Greene *et al.* 1988	16h:8h	540 to 1080	NI	NI
B.C. MEP 1988	14h:10h	NI	NI	NI
Poirier *et al.* 1988	16h:8h	≤800	cool white Fl.	NI

9. Temperature, Aeration, and Dissolved Oxygen During Test

Document	Water Temperature (°C)	Aeration	Minimum Oxygen
APHA *et al.* 1985	20	none	NI
ISO 1982	20 ± 2	NI	≥2 mg/L
ASTM 1984	20 ± 2	none	NI
OECD 1981	18 to 22 (±0.5)	none	>70% sat'n.
U.S. EPA 1982	20 ±1	none	≥60% sat'n.
Plotkin/Ram 1983	16 to 25	gentle if DO <40% sat.	≥40% sat'n.
The Netherlands 1980	19 ± 1	none	≥80% sat'n.
BHSC 1982	18 to 22 (±1.0)	none	≥2 mg/L
U.S. EPA 1985b	20	NI	≥60% sat'n.
Greene *et al.* 1988	20 ± 2	none	NI
B.C. MEP 1988	20 ± 2	none	NI
Poirier *et al.* 1988	20 ± 1	NI	NI

10. Adjustment of pH Prior to Test

Document	Treatment Specified
APHA *et al.* 1985	NI
ISO 1982	NI
ASTM 1984	NI
OECD 1981	do not adjust
U.S. EPA 1982	NI
Plotkin/Ram 1983	adjust to pH 7.0 if <6.0 or >8.0
The Netherlands 1980	adjust to control value if desired
BHSC 1982	do not adjust; repeat test at dilution water pH
U.S. EPA 1985b	NI
Greene *et al.* 1988	run parallel test with adjusted pH if <6 or >10
B.C. MEP 1988	NI
Poirier *et al.* 1988	if desired, run additional adjusted test

11. Biological Observations During Test

Document	Variable	Time (hours and other)
APHA *et al.* 1985	mobility	1 2 4 8 16+ daily
ISO 1982	mobility	24
ASTM 1984	death or mobility	24 48
OECD 1981	mobility	24
U.S. EPA 1982	mobility	3 6 12 24 48
Plotkin/Ram 1983	death	1 2 4 8 12 24 48
The Netherlands 1980	death	3 6 24 48
BHSC 1982	mobility	24
U.S. EPA 1985b	death or mobility	48
Greene *et al.* 1988	death, lethargy, floating	24 48
B.C. MEP 1988	death	24 48
Poirier *et al.* 1988	death, mobility, circling, floating	1 2 4 24 48

12. Monitoring Water Quality During Tests

Document	Variable*	Frequency (hours)
APHA *et al.* 1985	NI	NI
ISO 1982	DO	0 24
ASTM 1984	DO pH T cond hard SS alk	0 4 8 24 48
OECD 1981	DO pH T	0 24
U.S. EPA 1982	DO pH T	0 24 48
Plotkin/Ram 1983	DO	1 2 4 8 12 24 48
The Netherlands 1980	DO pH	0 48
BHSC 1982	DO pH	0 24
U.S. EPA 1985b	DO pH T	0 48
Greene *et al.* 1988	DO pH T cond hard alk	0 24 48
B.C. MEP 1988	DO pH	0 48
Poirier *et al.* 1988	DO pH cond hard	0 48

* DO = dissolved oxygen
T = temperature
cond = conductivity
hard = total hardness
SS = suspended solids
alk = total alkalinity

13. Test Endpoints

Document	Endpoints			Exposure-time (hours)		
APHA *et al.* 1985	EC_{50}			NI		
ISO 1982	EC_{50}			24		
ASTM 1984	EC_{50}	or	LC_{50}	24	and	48
OECD 1981	EC_{50}			24		
U.S. EPA 1982	EC_{50}			24	and	48
Plotkin/Ram 1983			LC_{50}			48
The Netherlands 1980			LC_{50}			48
BHSC 1982	EC_{50}			24		
U.S. EPA 1985b	EC_{50}	or	LC_{50}			48
Greene *et al.* 1988			LC_{50}			48
B.C. MEP 1988			LC_{50} or LT_{50}			≤48
Poirier *et al.* 1988	EC_{50}	and	LC_{50}			48

Appendix C

Procedure for Preparing YCT and Algal Food for Daphnid Cultures*

Preparing Digested Trout Chow

1. Preparation of trout chow requires one week. Use starter or No. 1 pellets.

2. Add 5.0 g of trout chow pellets to 1 L of deionized (Milli-Q™ or equivalent) water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation should be replaced during digestion. Because of the offensive odour usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.

3. At the end of the digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (e.g., Nitex™, 110 mesh). Combine equal volumes of supernatant from Cerophyll™ and yeast preparations (see following). The supernatant can be used fresh, or frozen until use. Discard the sediment.

Preparing Yeast

1. Add 5.0 g of dry yeast, such as Fleischmann's™, to 1 L of deionized water.

2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.

3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow (above) and Cerophyll preparations (see following). Discard excess material.

Preparing Cerophyll (Dried, Powdered Cereal Leaves)

1. Place 5.0 g of dried, powdered cereal leaves** in a blender. Dried, powdered alfalfa leaves obtained from health food stores have been found to be a satisfactory substitute.

2. Add 1 L of deionized water.

3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.

4. If a blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow to settle for 1 h. Decant the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations. Discard excess material.

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* from EPA 1989.

** Available as "Cereal Leaves" from Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri 63178 (800) 325-3010; or as Cerophyll™, from Ward's Natural Science Establishment Inc., P.O. Box 92912, Rochester, New York 14692-9012 (716) 359-2502.

Preparing Combined YCT Food

1. Mix equal (approximately 300 mL) volumes of the three foods described previously.

2. Place aliquots of the mixture in small (50 to 100 mL) screw-cap plastic bottles and freeze until needed.

3. Freshly-prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings, and is used for a maximum of two weeks.

4. It is advisable to measure the dry weight of solids in each batch of YCT before use. The food should contain 1.7 to 1.9 g solids/L. Cultures or test solutions should contain 12 to 13 mg solids/L.

***Preparing Food for Alga* (Selenastrum)**

A. Algal culture medium

1. Prepare five stock nutrient solutions using reagent-grade chemicals as described in Table C1.

2. Add 1 mL of each stock solution, in the order listed in Table C1, to approximately 900 mL of deionized water. Mix well after each solution is added. Dilute to 1L, mix well, and adjust the pH to 7.5 ± 0.1, using 0.1*N* NaOH or HCl, as appropriate. The final concentration of macronutrients and micronutrients in the culture medium is given in Table C2.

3. Immediately filter the pH-adjusted medium through a 0.45 μm pore diameter membrane at a vacuum of not more than 380 mm mercury, or at a pressure of not more than one-half atmosphere. Wash the filter with 500 mL deionized water prior to use.

4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels.

5. Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

B. Establishing and maintaining stock cultures of algae

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring 1 mL to each of several 250-mL culture flasks containing 100 mL of algal culture medium (prepared as described). The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.

2. The stock cultures are used as a source of algae to initiate "food" cultures (see following section). The volume of stock culture maintained at any one time will depend on the amount of algal food required for the daphnid cultures. Stock culture volume may be rapidly "scaled up" to several litres, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.

Table C1 Nutrient Stock Solutions for Maintaining Stock Cultures of Algae

Nutrient Stock Solution	Compound	Amount Dissolved in 500 mL Deionized Water
1	$MgCl_2 \cdot 6H_2O$	6.08 g
	$CaCl_2 \cdot 2H_2O$	2.20 g
	H_3BO_3	92.8 mg
	$MnCl_2 \cdot 4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg*
	$FeCl_3 \cdot 6H_2O$	79.9 mg
	$CoCl_2 \cdot 6H_2O$	0.714 mg**
	$Na_2MoO_4 \cdot 2H_2O$	3.63 mg***
	$CuCl_2 \cdot 2H_2O$	0.006 mg****
	$Na_2EDTA \cdot 2H_2O$	150.0 mg
2	$NaNO_3$	12.75 g
3	$MgSO_4 \cdot 7H_2O$	7.35 g
4	K_2HPO_4	0.522 g
5	$NaHCO_3$	7.50 g

* $ZnCl_2$ -- Weigh out 164 mg, dilute to 100 mL. Add 1 mL of this solution to Stock Solution #1.

** $CoCL_2 \cdot 6H_2O$ -- Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock Solution #1.

*** $Na_2MoO_4 \cdot 2H_2O$ -- Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock Solution #1.

**** $CuCl_2 \cdot 2H_2O$ -- Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock Solution #1.

Table C2 Final Concentration of Macronutrients and Micronutrients in the Culture Medium

Macronutrient	Concentration (mg/L)	Element	Concentration (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

Micronutrient	Concentration (μg/L)	Element	Concentration (μg/L)
H_3BO_3	185.	B	32.5
$MnCl_2 \cdot 4H_2O$	416.	Mn	115.
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160.	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300.	--	--

3. Culture temperature is not critical. Stock cultures may be maintained at 20 to 25°C in environmental chambers with cultures of other organisms if the illumination is adequate, i.e., continuous "cool-white" fluorescent lighting of approximately 4300 lux (400 foot-candles).

4. Cultures are mixed twice daily by hand.

5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three millilitres of seven-day old algal stock culture, containing approximately 1.5 x 10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 10 000 to 30 000 cells/mL in the new stock cultures, and care should be exercised to avoid contamination by other microorganisms.

6. Stock cultures should be examined microscopically weekly, at transfer, for microbial contamination. Reserve quantities of culture organisms can be maintained for 6 to 12 months if stored in the dark at 4°C. Every 4 to 6 months, it is advisable to prepare new stock cultures from "starter" cultures obtained from established, outside sources.

C. *Establishing and maintaining "food" cultures of algae*

1. "Food" cultures are started seven days prior to use in daphnid cultures. Approximately 20 mL of seven-day-old algal stock culture (described in the previous paragraph), containing 1.5 x 10^6 cells/mL, are added to each litre of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of approximately 30 000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other micro-organisms.

2. Food cultures may be maintained at 20 to 25°C in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 4300 lux).

3. Cultures are mixed continuously on a magnetic stir plate (with a medium-size stir bar) or in a moderately-aerated separatory funnel, or are mixed twice daily by hand*. Caution should be exercised to prevent the culture temperature from rising more than 2 to 3°C.

D. *Preparing algal concentrate for use as food for daphnids*

1. An algal concentrate containing 3.0 to 3.5 x 10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for approximately 2 to 3 weeks and siphoning off the supernatant.

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* If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees.

2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer, and used to determine the dilution (or further concentration) required to achieve a final cell count of 3.0 to 3.5 x 10^7/mL.

3. Assuming a cell density of approximately 1.5 x 10^6 cells/mL in the algal food cultures at seven days, and 100% recovery in the concentration process, a 3-L, 7- to 10-day culture will provide 4.5 x 10^9 algal cells. This number of cells will provide approximately 150 mL of algal cell concentrate for use as food for the daphnid cultures (Section 2.4.10).

4. Algal concentrate may be stored in the refrigerator for one month.

Appendix D

Logarithmic Series of Concentrations Suitable for Use in Toxicity Tests*

Column (Number of concentrations between 100 and 10, or between 10 and 1)**						
1	2	3	4	5	6	7
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3.2	10	18	25	32	37	42
1.0	4.6	10	16	22	27	32
	2.2	5.6	10	15	19	24
	1.0	3.2	6.3	10	14	18
		1.8	4.0	6.8	10	13
		1.0	2.5	4.6	7.2	10
			1.6	3.2	5.2	7.5
			1.0	2.2	3.7	5.6
				1.5	2.7	4.2
				1.0	1.9	3.2
					1.4	2.4
					1.0	1.8
						1.3
						1.0

* Modified from Rochinni *et al.* (1982).

** A series of five (or more) successive concentrations may be chosen from a column. Mid-points between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage by volume or weight, mg/L, or µg/L. As necessary, values may be multiplied or divided by any power of 10. Column 1 might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations (differing by a factor <0.3) should not be used. For effluent testing, there is seldom much gain in precision by selecting concentrations from a column to the right of column 3; the finer gradations of columns 4-7 might occasionally be useful for testing chemicals that have an abrupt threshold of effect.